

**POPULATION DIVERSITY IN  
*THEILERIA ANNULATA* IN TUNISIA**

**LEILA BEN MILED**

**Ph.D.  
University of Edinburgh  
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To DR E. Riley

with Thanks



**This presentation is entirely the product of my own efforts and the work on which it is based was my own except where specifically stated in the text and in the acknowledgements section.**

**Leïla Ben Miled**

**November, 1993.**

## DEDICATION

To my mother and father

with all my love.

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ABSTRACT OF THESIS (Regulation 3.5.10)

Candidate ..... Leila Ben Miled .....  
 C.T.V.M. University of Edinburgh, Easter Bush, Roslin, Scotland EH25 9RG .....  
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Tropical theileriosis, caused by the haemoprotozoan parasite *Theileria annulata*, is one of the major threats to cattle health in Tunisia. The aim of the study described in this thesis was to assess the extent of diversity in *T. annulata* parasite populations from within a single country in order to provide a better understanding of the epidemiology of the disease and biology of the parasite. The work also provided useful background information for vaccine development studies in Tunisia.

Different *T. annulata* stocks, isolated from different bioclimatic zones in Tunisia were characterised using monoclonal antibodies (MAbs), isoenzyme and DNA analyses and compared with each other and with a number of recognised laboratory stocks from other theileriosis endemic areas of the world.

The study comprises seven chapters. In the first, an introduction to the literature describing the parasite *T. annulata* and the disease it causes are presented and the epidemiology of tropical theileriosis is discussed with particular regard to the situation in Tunisia. Finally an overview on diversity in protozoan parasites, including *Theileria*, is given to emphasise the rationale behind the present study.

The next chapter details how the biological material, including 51 field isolates, was generated for the parasite life-cycle stages, sporozoites, piroplasms and schizonts.

In the third chapter the production of antischizont MAbs and their use as immunological markers to reveal differences between stocks of *T. annulata* is described.

The fourth chapter demonstrates the use of a biochemical marker, glucose phosphate isomerase isoenzyme to study diversity in *T. annulata* isolated in Tunisia.

In the fifth chapter, DNA probes generated from a genomic library of *T. annulata* piroplasms were analysed to select appropriate markers for characterisation of genomic variants of the parasite. One of these, the T17 probe, was used to define five variants which were obtained as cloned infected lymphoblastoid cell lines. Restriction mapping of these variants showed that deletion, point mutation and translocation might be common events in the *T. annulata* genome. It was also found that part of this probe comprises a cysteine protease gene which possesses some homology with a *T. parva* cysteine protease gene.

The sixth chapter analyses the diversity in the stocks isolated from Tunisia using the markers selected and described in the previous chapters. The diversity existing between isolates was analysed at several levels; within isolates from one country, one bioclimatic zone, one site of isolation, one farm or, finally one animal and the results show that tremendous polymorphism exists. Experiments performed to assess the reliability of the methods used are also presented.

In the final chapter, investigations into the infectivity, virulence and immunity stimulated by individual cloned infected cell lines compared to a mixture of the same clones are described. The implications of the results of this study are discussed in relation to the selection of appropriate vaccines for use in the field. In addition, DNA from parental cell lines and the clones derived from them, obtained by tick transmission from the calves used in the infectivity study were analysed by southern blotting using DNA probes. The results showed that parasite clones of novel, non-parental genotype were obtained in the progeny and recombination between the parental characters was demonstrated. By localising the different DNA probes on the chromosome of *T. annulata* it was possible to show that recombination was due either to independent assortment between non-homologous chromosomes or crossing over between homologous chromosomes. These results give evidence for sexual reproduction in *T. annulata*. This, together with the findings that deletion, point mutation and translocation might occur in the *T. annulata* genome give a new approach to studying genetics in *Theileria* and help to explain the marked polymorphism found in this study.

## ABBREVIATIONS

ALD	Aldolase
BPA	Bovine plasma albumin
CPM	Counts per min
CTL	Cytotoxic T-lymphocytes
CTVM	Centre for Tropical Veterinary Medicine
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ENMV	Ecole Nationale de Medecine Veterinaire
F <sup>*</sup>	Fragment
FAO	Food and Agriculture Organisation
FCS	Fetal calf serum
GALDH	Glyceraldehyde phosphate dehydrogenase
GF	GUTS filtrate
GPI	Glucose phosphate isomerase
GUTS	Ground up tick supernatant
HBSS	Hanks balanced salt solution
HGPRT	Hypoxanthine-guanine phosphoribosyl transferase
IEF	Isoelectric focusing
IFAT	Indirect fluorescent antibody test
Ig	Immunoglobulin
IPA	Institut Pasteur d'Algerie
IPT	Institut Pasteur de Tunis
Kb	Kilobases
kDa	Kilodaltons
LB	Luria broth
LD	Lethal dose
LDH	Lactate dehydrogenase
LMA	Low melting agarose
MAb	Monoclonal antibodies
MEM	Minimum essential medium
MGP	Methyl green pyronin
MHC	Major histocompatibility complex
MLR	Mixed leucocyte reaction
NK	Natural killer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PEG	Polyethylene glycol
PFGE	Pulse field gel electrophoresis
PL	Platelets
PBM	Peripheral blood mononuclear cells
RPMI	Roswell Park Memorial Institute medium
RBC	Red blood cell
RNA	Ribonucleic acid

SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SG	Salivary glands
<i>T.a</i>	<i>Theileria annulata</i>
<i>TaT</i>	<i>T.annulata</i> Tunis
<i>TaTu</i>	<i>T.annulata</i> Tunisia
t.e.	tick equivalent
TBE	Tris borate EDTA buffer
TE	Tris EDTA buffer
WBC	White blood cell
WUMP	Wellcome Unit of Molecular Parasitology

## ***CHAPTER ONE***

### ***GENERAL INTRODUCTION AND LITERATURE REVIEW***

## 1.1 INTRODUCTION

This chapter is divided in four parts. Firstly an overview on the parasite, *Theileria annulata* and the disease it causes, tropical theileriosis is given. Secondly, the epidemiology of tropical theileriosis in Tunisia is presented since the biological material used in this thesis was mainly collected from this country. Since this study focuses on diversity in *T.annulata*, in the third part of the general introduction, diversity within *Theileria* spp as well as amongst other protozoan parasites is presented in order to summarise the knowledge in this particular field and to contribute to the interpretation of the results presented in this thesis. Finally, the conclusion comprises an assessment of this earlier work on diversity and a summary of the study objectives which examine diversity of *T.annulata* in Tunisia applying the most relevant of these techniques.

## 1.2 THEILERIA ANNULATA AND TROPICAL THEILERIOSIS

### 1.2.1 History

In 1904, E.Dschunkowsky and I.Luhs first described tropical bovine piroplasmosis, a fatal disease affecting cattle in Transcaucasia, in the former U.S.S.R. (Dschunkowsky and Luhs, 1904). They associated the disease with the presence of an haemoprotezoan parasite which was subsequently named *Piroplasma annulatum* (Bettencourt *et al*, 1907). Blood smear examination revealed parasites similar but not identical to those described by Koch (1906) as the causative agent of what emerged as East Coast fever in East and Southern Africa and was named *Theileria parva* (Theiler, 1904). The presence of the schizont stage of *P.annulatum* in the bovine host led Bettencourt *et al* (1907) to remove the parasite from the genus *Piroplasma* and place it in the genus *Theileria*. It subsequently became known as *Theileria annulata*.

The disease is widely distributed from Morocco in the West to China in the East (see Section 1.2.3). As a result it was initially reported from many countries with confusing terminology. Authors frequently referred to it either as a piroplasmosis or as a variety of *T.parva*. Ducloux (1905) referred to the disease as "Piroplasmose bacilliforme du boeuf" (bovine bacilliform piroplasmosis) in Tunisia. In the Sudan it was also described as a

piroplasmosis by Balfour (1908). Carpano (1912, 1915) reporting from Eritrea and Libya, considered the causative agent to be "*Theileria parva*", and named the disease "La febbre Della Costa Meditteranea. Piroplasmosis tipo 'parvum' nei bovine del basso bacino del Mediterraneo" (The fever of the Mediterranean coast. Piroplasmosis of 'parvum' type of the bovine from the low Mediterranean basin). Pricolo (1914, 1915, 1921) was also of this opinion and described the disease in Tunisia as "Piroplasmosi da *Theileria parva* nell' Africa del Nord" (*Theileria parva* piroplasmosis of North Africa) (All cited by Neitz, 1957). In 1919, Sergent and collaborators suggested that tropical theileriosis was different from the other bovine piroplasmosis in Algeria caused by either *Piroplasma bovis* or *Babesiella bigemina* (Sergent *et al*, 1919). However they referred to the parasite responsible as *T.dispar* rather than *T.annulata*. On the recommendation of the French colonial government of Algeria who recognised tropical theileriosis as a major constraint to the cattle industry, a team of workers, led by E.Sergent of the Institut Pasteur d'Algerie, began intensive studies on the parasite and the disease caused by it. These studies have provided much of the fundamental research results that have been the basis for subsequent work on *T.annulata*. The summary of 30 years of work by this team is reported in a volume entitled "Etudes sur les piroplasmoses bovines" (Studies on the bovine piroplasmoses) (Sergent *et al*, 1945) which includes 50 papers on *T.annulata*.

Other names, such as *T.turkestanica* and *T.sergenti*, were also used to describe the parasite responsible for theileriosis occurring in Turkistan and East Asia respectively (Neitz, 1957). The former was almost certainly *T.annulata*, but controversy still surrounds the identity of the latter. In his review, Neitz (1957) stated the cause of tropical theileriosis to be *T.annulata* with *T.dispar*, *T.turkestanica* and *T.sergenti* as synonyms. An attempt to reclassify *T.annulata* as *Gonderia annulata* was made by Neitz and Jansen (1956), retaining only *T.parva* in the family *Theileriidae* (Du Toit, 1918) as a single genus and single species. This classification was based on the view prevalent at that time that *T.parva* was not thought to undergo intraerythrocytic replication in contrast to parasites in the genus *Gonderia*. This reclassification was abandoned when Neitz (1964) showed that multiplication of piroplasms within red blood cells also occurs in *T.parva*.

Sergent and co-workers produced convincing evidence that the parasite causing East Coast fever, *T.parva*, and that responsible for theileriosis in North Africa, *T.dispar*, were two distinct species (Sergent *et al*, 1927). They used several criteria. Firstly, a study of the morphology of the parasite stage within erythrocytes showed that more rod-shaped forms occurred in *T.parva* than in *T.dispar* in which, oval annular forms predominated. This alone was insufficient evidence to prove that the two species were distinct, but the results of tick transmission experiments (xenodiagnosis) showed that while *Rhipicephalus appendiculatus*, the main vector of *T.parva* in Eastern and Southern Africa, transmits *T.parva*, it failed to transmit *T.dispar*. In addition cross-immunity trials demonstrated that *T.parva* did not protect against *T.dispar* and vice versa. *T.dispar* from Algeria, and *T.annulata* from other countries were only accepted as being the same species when cross-immunity experiments showed that the parasites were very closely related immunologically and could be considered identical (Sergent *et al*, 1935, 1937). This conclusion was supported by the fact that the Algerian blood vaccine comprising the Kouba strain (Sergent *et al*, 1932) was used successfully in Palestine to immunise imported cattle (Anonymous, 1949).

### 1.2.2 Classification

A revised classification of *Theileria* has been given by Levine (1988) as follows:

Phylum	Apicomplexa
Class	Aconoidasida
Order	Piroplasmorida
Family	Theileriidae
Genus	<i>Theileria</i>

The most important *Theileria* species are listed in Table 1.1 (Dolan, 1989). They affect wild and domestic cattle, sheep and goats. Clarification of the identity of the members of the *T.orientalis*/*T.buffeli*/*T.sergenti* group is still required to resolve their taxonomy.

Since the present study is concerned with *T.annulata* affecting domestic cattle in Tunisia, this review will focus on this species with reference to other protozoa, particularly *T.parva*, where appropriate.

Table 1.1      The important *Theileria* parasites of domestic animals (T.T.Dolan, 1989).

Parasite	Animal	Vector	Disease	Distribution/comments
<i>Theileria annulata</i> (Dschunkowsky and Luhs, 1904)	Cattle, Domestic buffalo ( <i>Bubalus bubalis</i> )	<i>Hyalomma</i> spp.	Tropical or Mediterranean theileriosis	North Africa, southern Europe, Middle East, India and southern USSR
<i>Theileria camelensis</i>	Camels	Unknown	Unknown	Africa and parts of the USSR
<i>Theileria hirci</i> (Dschunkowsky and Urodschewich, 1924)	Sheep, Goats	<i>Hyalomma</i> spp.	Malignant theileriosis of sheep and goats	North Africa, south-eastern Europe, Near and Middle East and southern USSR
<i>Theileria mutans</i> (Theiler, 1906)	Cattle, Buffalo ( <i>Syncerus caffer</i> ), Sheep?	<i>Amblyomma</i> spp.	Benign theileriosis	Sub-Saharan Africa and possibly the Caribbean
<i>Theileria orientalis</i> (Yakimoff and Soudatschenkoff, 1931)	Cattle	<i>Haemaphysalis</i> spp. <i>Amblyomma</i> spp. in Africa		The distinction between <i>T.orientalis</i> & <i>T.sergenti</i> (Yakimoff and Dekhtereff, 1930) arises because the latter is less pathogenic worldwide but pathogenic in the USSR, Japan and Far East
<i>Theileria parva</i> (Theiler, 1906)	Cattle, Buffalo and domestic buffalo experimentally	<i>Rhipicephalus</i> <i>appendiculatus</i> <i>R.rambesziensis</i>	East Coast fever ( <i>T.p.parva</i> ) Corridor disease ( <i>T.p.lawrencei</i> ) Rhodesian theileriosis ( <i>T.p.bovis</i> )	East and Central Africa. The trinomial nomenclature for subspecies is adopted for convenience. <i>T.p.parva</i> and <i>T.p.bovis</i> transmitted between cattle, <i>T.p.lawrencei</i> transmitted from buffalo to cattle.
<i>Theileria taurotragui</i> (Martin and Brocklesby, 1960)	Cattle, other Bovidae	<i>Rhipicephalus</i> spp.		Africa. Occurs naturally in antelopes, particularly eland, and can be pathogenic occasionally in cattle.
<i>Theileria velifeera</i> (Uilenberg, 1964)	Cattle, Buffalo	<i>Amblyomma</i> spp.		Sub-Saharan Africa and the Caribbean (apathogenic)

Other *Theileria* spp. of small ruminants which are non-pathogenic but which might confuse diagnosis are *T.ovis*, *T.recondita* and *T.separata*.



### 1.2.3 Distribution

Topical theileriosis is widespread from Morocco, Spain and Portugal in the West, along the Mediterranean littoral, including Tunisia, through the Near and Middle East, the Indian subcontinent and Southern Republics of the old U.S.S.R. to China (Purnell, 1978). Where the distribution has been described as extending to Far Eastern countries and into Malaysia, this probably includes the distribution of *T.sergenti*/*T.orientalis* (Dolan, 1989). A recent description of the distribution of *T.annulata*, *T.parva* and *T.sergenti* (Fig. 1.1) shows the absence of tropical theileriosis in Libya (McHardy, 1991), although the disease was previously reported in Libya by Carpano in 1915 and Pricolo in 1914, 1915 and 1921, as cited by Neitz (1957). The disease was reported to occur in China by Purnell (1978). The extent of *T.annulata* distribution in China and the former U.S.S.R. is not well documented (Dolan, 1989).

The disease represents a threat to the countries of Northern Europe not only from North Africa but also from Portugal, Spain, France and Italy in the South, as well as Greece and Turkey, in the East where the disease is widespread. Eastern countries such as Japan only report the presence of parasites of the *T.sergenti*/*T.orientalis* group, which seem to be replacing *T.annulata* in this region. However the exact distribution of the two parasites remains uncertain.

The distribution of *T.annulata* is generally distinct from that of *T.parva* (Fig. 1.1). Only in the Sudan, are both parasites found, the former in the North and the latter in the South. It is the only geographical region where both diseases might overlap due to cattle movement (S.Morzaria, personal communication).

### 1.2.4 Host Susceptibility

*T.annulata* is a tick-borne haemoprotozoan parasite causing the disease, tropical theileriosis in domestic cattle (*Bos taurus* and *Bos indicus*) and Asian buffalo (*Bubalus bubalis*). Steuber *et al* (1986) examined the host range of *T.annulata* *in vitro* and demonstrated that the sporozoites neither entered nor attached to peripheral blood mononuclear cells (PBM) from horse, rabbit, mouse, hamster or man. This study confirmed

**Figure 1.1**     Distribution of important forms of bovine theileriosis worldwide (McHar-  
1991)

# Distribution of Important Forms of Theileriosis



that the host range of *T.annulata* is limited to the bovid family. Indeed lymphocytes from all the bovidae examined were infected by sporozoites of *T.annulata*. The PBM used were from cattle (*Bos taurus*), African buffalo (*Syncerus caffer*), Asian buffalo (*Bubalus bubalis*), goat and sheep. It is worth noting that the two latter species were not susceptible to *T.parva* infection *in vitro* (Stagg *et al*, 1983). These experiments were carried out to determine the host range of the parasite and also to investigate the possibility of using a laboratory model to study the disease. Up to now experimental infections of *T.annulata* have been carried out in bovines (*Bos taurus* or *Bos indicus*) since no other species was found to be a satisfactory model for *T.annulata* infection.

Finally *T.annulata* has been transmitted experimentally to the American Bison (Carpano, 1937) and to goats (C.G.D.Brown and L.Bell-Sakyi, unpublished data).

#### 1.2.5 Transmission

In 1928, Sergent and co-workers demonstrated that *T.annulata* in Algeria was transmitted by an ixodid tick, *Hyalomma mauretanicum* (*H.detrutum*, Schulze, 1919) (Sergent *et al*, 1928). This was the first time that a tick of this genus had been shown to be the vector of an infectious disease. In 1936, the same authors described the developmental stages undergone by the parasite within the invertebrate tick vector (Sergent *et al*, 1936a). Since then, several studies have shown the role of different *Hyalomma* species in transmitting the disease within the cattle population (Robinson, 1982). Barnett (1977), in a review on *Theileria*, reported that *H.detrutum* was the most efficient vector. However, Warnecke (1978) showed, in a survey carried out in Turkey, that although *Theileria* was found in *H.detrutum*, *H.marginatum*, *H.anatolicum excavatum* and *H.a.anatolicum*, the last-mentioned was the most highly infected and frequently found species. *H.a.excavatum* and *H.asiatium* (Middle East, India, Southern States of the former U.S.S.R.) have transmitted *T.annulata* experimentally (Barnett, 1977). The natural vector of the disease in these countries is *H.a.anatolicum*.

*Hyalomma* ticks may feed on different hosts as larvae, nymphs and adults. *H.a.anatolicum* feeding on cattle is usually a three-host tick whereas *H.detrutum* is a two-

host tick (Sergent *et al*, 1945), of which the larvae and nymphs feed on the same host. Ticks pick up *T.annulata* infection during their feed at one stage and transmit the disease transtadially at the next stage to another bovine. Although it was reported by some authors (Ray, 1950; Dipeolu and Ogunji, 1977), transovarian transmission is not generally accepted for ticks of the genus *Hyalomma*. Natural transmission is only known to occur through *Hyalomma* ticks, although intra-uterine transmission has been reported (Sergent *et al*, 1937). Artificial transmission using infected blood was shown to be possible in early studies (Sergent *et al*, 1939) and has enabled researchers to maintain the parasite by serial passage through cattle. *T.annulata* infection can also be transmitted by inoculation of *in vitro* cultured transformed lymphoblastoid cells (Tsur and Pipano, 1966) and this has provided a basis for the use of culture cells as vaccine material.

The standardisation of infection using tick material was made possible by the preparation of ground up ticks or their salivary glands in large quantities (Brown *et al*, 1973). The stabulates cryopreserved in glycerol (see Chapter two, Section 2.2.4.4) in aliquots offered the advantage of reproducible and quantified (as tick-equivalent, t.e) infections.

### 1.2.6 Life cycle

The development of *Theileria* spp in both invertebrate and vertebrate hosts has been reviewed recently by Norval *et al* (1992) who have summarised most of the contributions to the present understanding of the life cycle. Figure 1.2 illustrates the life cycle seen in North Africa under seasonal conditions where the two host tick *H.detrutum* is the primary vector.

The infection of the bovine host is initiated during adult tick feeding when sporozoites inoculated with tick saliva enter leucocytes. Sporozoite entry into the target cell is very rapid and occurs within a few minutes (Jura *et al*, 1983). Following sporozoite entry, the parasite develops into the uninucleate trophozoite stage which then undergoes nuclear division to form the multinucleate macroschizont. Trophozoites can be detected in cells, two hours after infection of PBM *in vitro* (C.G.D.Brown, personal communication). As the trophozoite develops into the macroschizont, the host cell becomes transformed and begins to divide in synchrony with division of the macroschizont producing two infected daughter

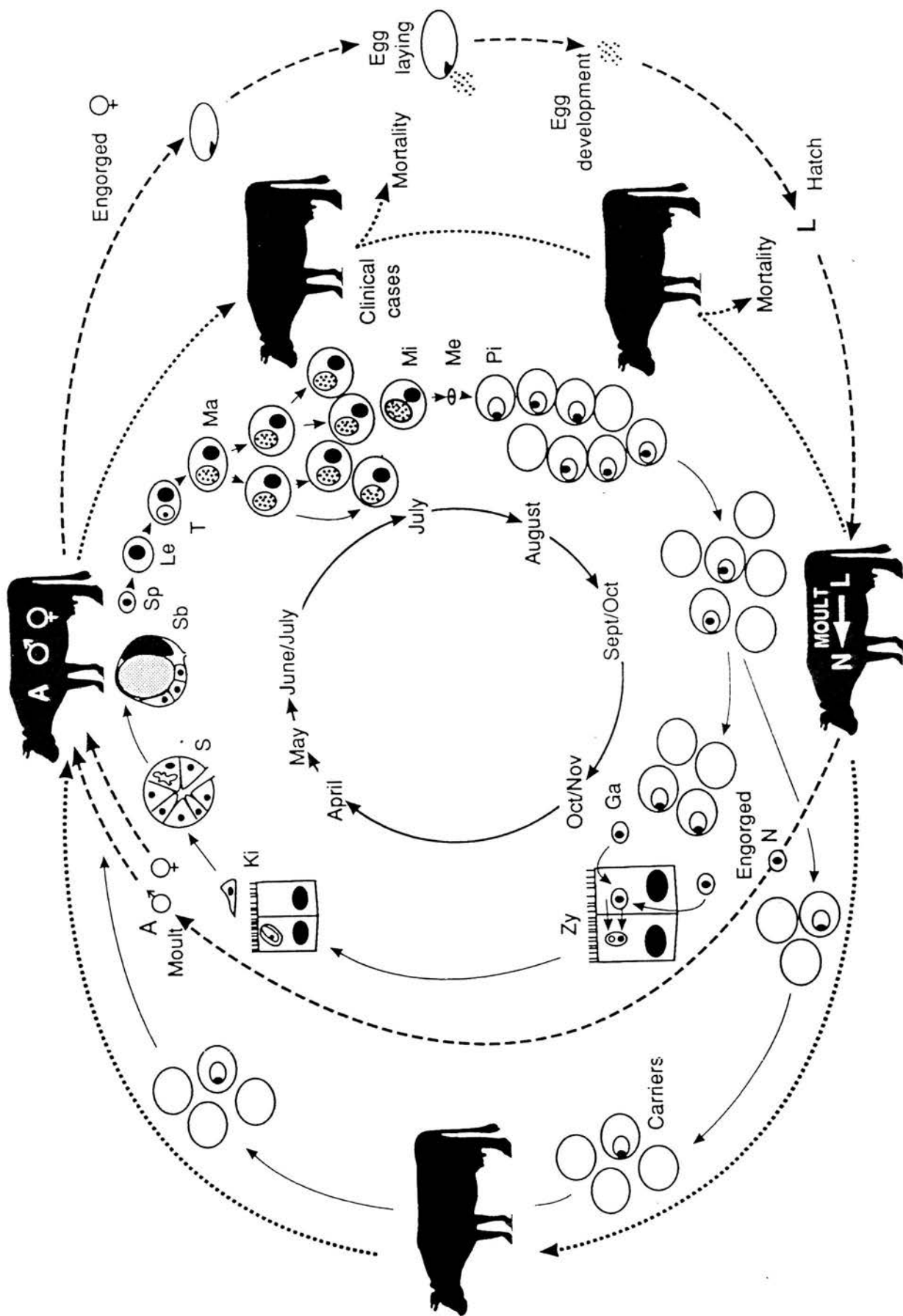
**Figure 1.2** Life cycles of *Theileria annulata* and *Hyalomma detritum* (Flach, 1991).

Abbreviations for *Theileria*:-

Le	Leucocyte
T	Trophozoite
Ma	Macroschizont
Mi	Microschizont
Me	Merozoite
Pi	Piropiasm
Ga	Gamete
Zy	Zgyote
Ki	Kinete
S	Salivary gland
Sb	Sporoblast
Sp	Sporozoite

Abbreviations for tick:

La	Larvae
N	Nymph
A	Adult



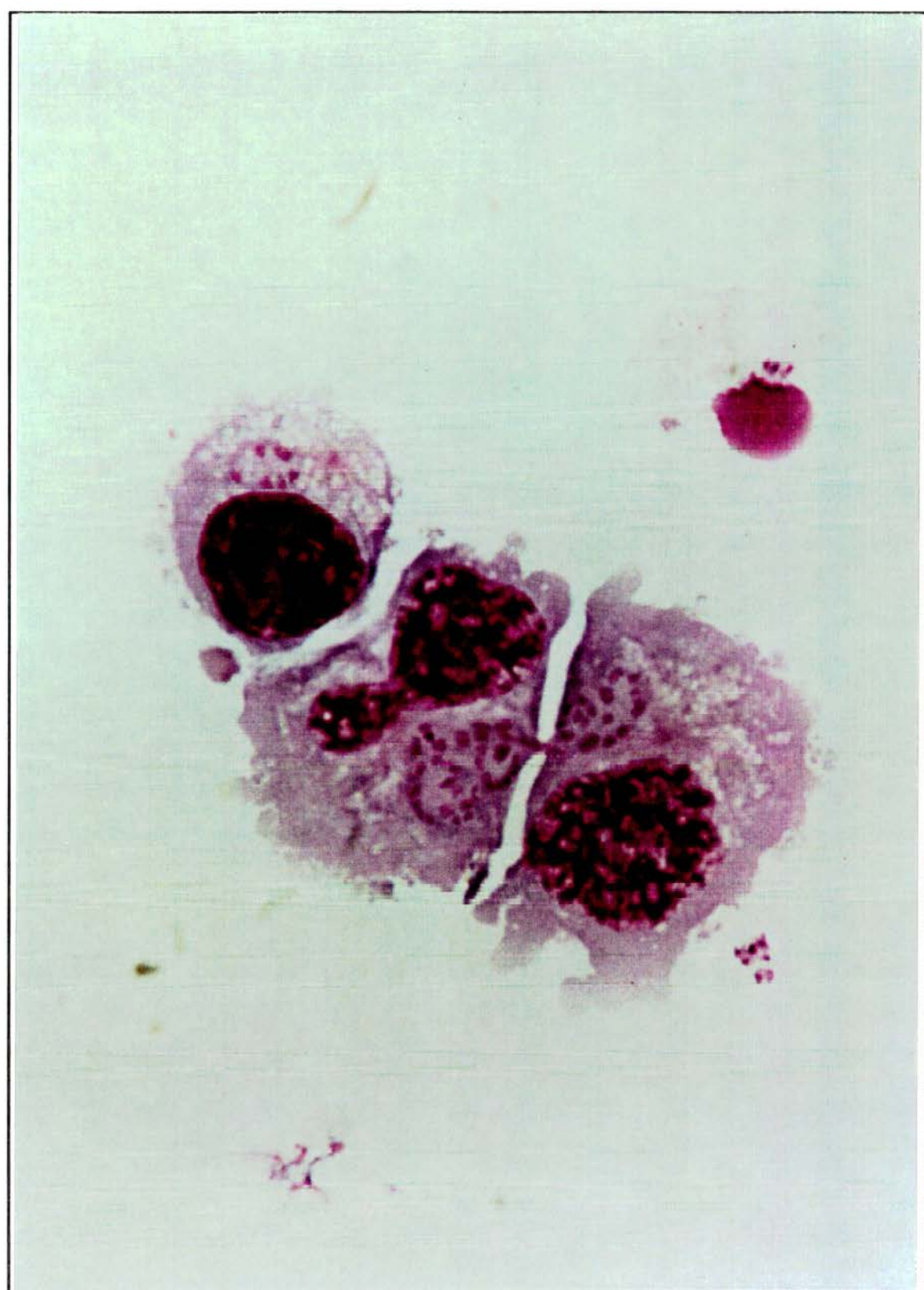


cells (Fig. 1.3) (Hulliger *et al*, 1964). The mechanism of transformation of the infected cells has not yet been explained, although a study of Dyer and Tait (1987) suggested the involvement of oncogenes. The phenomenon is reversible through the use of theilericidal drugs which kill the schizont *in vitro* resulting in death of the host cells a few days later (Pinder *et al*, 1981). This clonal expansion of infected cells is accompanied, in the animal, by spreading of infected cells from the lymph node draining the site of parasite inoculation throughout the body as in *T.parva* (De Martini and Moulton, 1973). After undergoing several divisions, a proportion of the macroschizonts differentiate into microschizonts in which a larger number of nuclei can be seen. The microschizont may occupy the whole cytoplasm of the parasitised cell and essentially comprises the differentiation of the macroschizont into uninucleate merozoites. This process involves the development of a distinct membrane surrounding individual schizont nuclei and the migration of these to the periphery of the schizont (Wilde, 1967). Merozoites are released by disruption of the host cell plasma membrane (Shaw and Tilney, 1992). They then invade host erythrocytes where they are known as piroplasms. The development within erythrocytes has been studied *in vitro* by Conrad *et al* (1985). Intraerythrocytic division was frequently observed whereas release of piroplasms and reinvasion of fresh erythrocytes could not be demonstrated with *T.annulata* using this culture technique. Intraerythrocytic *T.annulata* piroplasms are pleomorphic with annular forms predominating (Sergent *et al*, 1936b). The piroplasm stage represents the stage infective for the vector tick which ingests them with blood during engorgement.

The life-cycle of *T.annulata* in the two-host tick, *H.detritum* was first described by Sergent *et al* (1936a). Schein (1975) and Schein and Friedhoff (1978) described it in the tick vector *H.a.excavatum*. Koch (1906) was the first to observe the ray bodies or "strahlenkorper" of *T.parva* in the gut of engorged *R.appendiculatus* ticks. Schein (1975) also showed their presence in *H.a.excavatum* ticks infected with *T.annulata*. These structures have up to four flagella-like projections and a slender posterior pole. They are considered to be microgamonts as opposed to spherical stages or macrogamonts also present in the lumen of the gut (Norval *et al*, 1992). Zygotes are formed in the tick gut after syngamy



**Figure 1.3** Division of the macroschizont producing two infected daughter cells



of the two gametes. This stage and the succeeding kinete form are thought to be the only diploid forms during the parasite life cycle.

Sexual reproduction within the tick was strongly suspected by Irvin and Boarer (1980) and it is only recently that some evidence has been given for a sexual cycle in *T.parva* (Morzaria *et al*, 1992b). By showing that recombinant parasites of mixed parental genotypes can be obtained from ticks fed on animals infected with two distinct parental stocks, the study of Morzaria *et al* (1992b) confirmed the sexual cross between stocks and therefore provided more evidence for sexual reproduction in *T.parva*. Similar studies have not yet been reported for *T.annulata*.

The zygote transforms into a motile kinete which passes through the gut wall and invades the tick salivary glands towards the end of tick moult when the salivary gland cells are immature. The single kinete transforms into a sporoblast and undergoes multiple division. At this stage maturation is delayed until sporogony is stimulated by tick feeding or high temperature incubation (Samish, 1977). The parasitised acinar cell undergoes marked enlargement due to the parasite forming a multinucleate syncytium occupying most of the cell cytoplasm (Fawcett *et al*, 1985). During tick feeding there is a rapid development of the syncytium which gives rise to thousands of uninucleate sporozoites by cytoplasmic fission (approximately 40,000 per acinus) (Young *et al*, 1992). It is at this stage that the nuclear reduction is thought to happen with the parasite becoming haploid again (Gauer *et al*, 1993).

Several studies have been carried out on specific stages of the *Theileria* life-cycle, to provide a better understanding of parasite biology.

The nature of the bovine infected cells in *T.annulata* is controversial. *In vitro* studies showed that B-lymphocytes and monocytes are much more receptive to the parasite than T-lymphocytes (Spooner *et al*, 1989), the latter being the principal target for *T.parva* (Morrison *et al*, 1989). Spooner and Brown (1980) showed that infected cells *in vitro* stop expressing a number of cell markers, including those of monocytes and B-cells. Few studies have been done on the identity of the infected cell *in vivo*, in *T.annulata* infection, and this is yet to be determined.

Entry of sporozoites into host cells has also been investigated. A surface sporozoite

antigen of *T.annulata*, antibodies to which neutralise the sporozoite infectivity *in vitro* (Williamson *et al*, 1989), might be involved in the attachment of the infective stage to the cell during invasion (Hall, 1988).

A study on *T.parva* by Shaw *et al* (1991) showed that monoclonal antibodies (MAb), reactive with MHC class I molecules and  $\beta 2$  microglobulin can inhibit sporozoite entry in contrast to MAbs reactive with MHC class II molecules or common pan-leucocyte surface antigens which have no effect. This result indicates that MHC Class I molecules might have an important role in sporozoite entry in *T.parva*. However such a study has not been carried out yet for *T.annulata* and, since the target cells are different, the processes of entry might also differ between the two parasites.

The synchrony of division of the host cell and the parasite has been demonstrated from studies *in vitro* by Hulliger *et al* (1964). The same authors showed that this particular phenomenon allows the establishment of the cells *in vitro* as a continuously growing lymphoblastoid cell line. The mechanisms involved in the immortalisation of *Theileria*-infected cells are still unknown. This particular characteristic, however, has been invaluable in studies carried out on *Theileria* spp, since it provides continuously growing cell lines expandable *in vitro* which, similar to Epstein-Barr virus infected cells or cells activated by a carcinogen or oncogene, do not need any growth or stimulating factors (Hulliger *et al*, 1964).

A few studies have been carried out on the merozoite stage (Glascodine *et al*, 1990; Shiels *et al*, 1992). They have confirmed earlier work (Hulliger *et al*, 1966) which showed that *T.annulata* merozoites could be produced *in vitro* by cultivating schizont-infected cell lines at 41°C for several days. The resultant merozoites, however, were only able to infect erythrocytes *in vitro* at very low levels which suggests that important factors might intervene *in vivo* to facilitate this process (Dickson, 1993). The mechanism of penetration of *Theileria* merozoites into erythrocytes has not yet been established but it might, as for other protozoan parasites, involve the apical complex and rhoptries.

Young *et al* (1992) discuss factors which may control the dynamics of *Theileria* transmission basing their hypothesis on observations on *T.parva*. The authors showed that

temperature and humidity control the duration of tick survival and the ability to transmit parasites, the latter being enhanced by high temperatures. They explained the higher level of *Theileria* infection in female ticks compared to male ticks by the greater number of acini in the female salivary glands. In the same way, more sporozoites are produced in adult ticks than in nymphs which might be attributed to the same reason. Young *et al* (1992) also reported that cattle with high parasitemias (>30%) due to *T.parva* infection produce ticks with relatively lower infection rates than cattle with lower parasitemias. Incubation of infected adult ticks at high temperature (37°C) will also induce sporoblast maturation into infective sporozoites but at a much lower rate at any moment in time than in feeding ticks, resulting in a smaller number of sporozoites being produced at that time (Reid and Bell, 1981).

### **1.2.7 Importance of the disease**

In the early years of this century, bovine piroplasmosis, mainly due to *T.annulata* but also to other parasites such as *Babesia bovis*, *B.bigemina*, *Anaplasma marginale* and "*T.mutans*", (the latter probably being a parasite of the *T.buffeli/T.orientalis* group) was considered to be a major limitation to the development and upgrading of the cattle population by the introduction of improved European breeds, in the French colonies of North Africa, thus affecting the economic prosperity of "la jeune France Africaine" (the young African France) (Anonymous, 1949).

In endemic areas where the disease regularly occurs it normally induces minimal clinical reactions but probably has some effect on productivity. Animals are exposed to the parasite early in their life and either die or become immune and their immunity is maintained through constant rechallenge. However, in newly introduced animals in endemic areas, the disease is highly pathogenic causing 40-60% mortality (Brown, 1990). The presence of theileriosis has greatly interfered with crossbreeding programmes to upgrade the indigenous cattle population as reported by Adler and Ellenbogen (1935), Sergeant *et al* (1945), Matikashvili (1956) and Neitz (1957). Around 250 million cattle are at risk of the disease in endemic regions (Brown, 1990). Few studies have been carried out to estimate the economic

cost of theileriosis. Flach (1991) in a study in Morocco evaluated the losses caused by the disease, which included mortality, cost of treatment and reduced weight and milk production, at approximately £3,500 per 100 cattle per year on farms in endemic areas. The costs became more significant when improved breeds were introduced into these farms. The same study reported that, of 72 heifers of Holstein breed imported into Morocco at a price of £1,000 each, three died from theileriosis despite regular tick control. Brown (1990) has estimated the losses to India from *T.annulata* infection to be 10% of the contribution of livestock to the Gross National Product per year.

### **1.2.8 Immunity**

#### **1.2.8.1 Humoral response**

Attempts to prevent theileriosis using immune serum in passive transfer experiments have been unsuccessful (Sergent *et al*, 1945, Muhammed *et al*, 1975). This is despite claims that cattle were protected against *Theileria* infection by the use of immune serum (Robson *et al*, 1961). These findings have not been confirmed. Cunningham *et al* (1989) showed that calves, born to immune cows which had a significant level of anti-*Theileria* colostral antibodies, were susceptible to *T.parva* infection.

By using the indirect fluorescent antibody test (IFAT), BurrIDGE and Kimber (1972) were able to show that antibodies reacting with the schizont and piroplasm stages were always found in cattle which had been exposed to *Theileria* infection. Thus the IFAT became the standard serological test for *Theileria* antibodies. Following these findings, attempts were made to protect cattle by transfer of extracted schizont or piroplasm antigens. These indeed produced a similar level of antibody (detectable on the IFAT) to that observed in immune cattle but they did not play any role in protective immunity since the animals were fully susceptible on challenge (Wagner *et al*, 1974). Antibodies produced in immune animals do not react with the surface of either the schizont-infected cells (Duffus *et al*, 1978) or the piroplasm-infected erythrocytes (Hall, 1988).

None of the workers mentioned above tested for antibodies reacting with the sporozoite stage in *Theileria* infected cattle. However Gray and Brown (1981) showed that



immune bovine serum from cattle exposed on several occasions to *T.annulata* sporozoites neutralises the infectivity for PBM on sporozoites *in vitro*. In a study on *T.parva*, Musoke *et al* (1982) reported similar findings and noticed that the activity was enhanced when the animals had been exposed to repeated challenge with large numbers of infected ticks. Preston and Brown (1985) noticed that *T.annulata* immune sera, from cattle receiving repeated inoculation of viable sporozoites, also delayed the development of trophozoites into schizonts *in vitro*.

In agreement with trials *in vivo* which showed an absence of cross-immunity between *T.annulata* and *T.parva* (Sergent *et al*, 1945), there was no cross-reactivity between immune *T.annulata* and *T.parva* anti-sera in neutralising the infectivity of sporozoites *in vitro* (Gray and Brown, 1981). In the same study the authors showed that *T.annulata* immune serum raised against one stock of *T.annulata* was able to neutralise the infectivity *in vitro* of heterologous sporozoite stocks.

MAB have been raised against the sporozoite stage. Some of these MAB showed effective inhibition of sporozoite infectivity *in vitro* in both *T.annulata* (Williamson *et al*, 1989) and *T.parva* (Dobbelaere, 1984). These MAB have been the starting point of work towards identification of sporozoite antigens, with a degree of homology found between the two species, which might be relevant in the development of a multi-component subunit vaccine (Williamson *et al*, 1989; Musoke *et al*, 1992; Nene *et al*, 1992).

Humoral immunity is not considered to play a primary role in the protection of animals which have recovered from theileriosis. Evidence for this was given by the studies mentioned above and by Pipano (1981) who observed that the level of serum antibody titre does not relate to the degree of protection in cattle exposed to *T.annulata* parasites. It must be remembered, however, that this antibody titre is against the schizont (detected by IFAT). However, the identification of immunogenic antigens from all parasite stages using either immune serum or MAB may help in the design of defined subunit vaccine. These antigens might induce strong humoral responses in the bovine host to different stages in the parasite life-cycle, such as inhibition of the sporozoite and merozoite entry into their respective target cells. The first would prevent animals from suffering clinical disease and the second might

prevent the animals from suffering from the anaemia common in tropical theileriosis and from becoming carriers. These two situations have different implications on the epidemiology of the disease. In the first, there would be reduction of the disease incidence while eradication of the parasite would be the consequence of the second.

The ability of immune sera to block sporozoite invasion *in vitro* means that the sporozoite antigens are a target for the immune response in the bovine host. This could be used for the development of a multi-component subunit vaccine in that, if an effective response is established in cattle against the sporozoite stage, the host immune system could eliminate sporozoites more successfully and thus reduce the number of schizont-infected cells derived from parasites that evaded the first humoral barrier.

There is a correlation between the infective dose of sporozoites and the severity of the disease in *T.annulata* (Gill *et al*, 1981) as with *T.parva* (Cunningham *et al*, 1974). It was also shown by Preston and Brown (1985) that the humoral response in *T.annulata* infected cattle plays an important role in resistance to subsequent challenge. Hence protective sporozoite antigens inducing a strong humoral response could represent an important component of a subunit vaccine for immunising cattle in endemic areas. Other components which would be included in such vaccines would target the schizonts and the derived merozoites.

Studies on *T.annulata* have targeted such molecules on these different life-cycle antigens. The antigen identified by Williamson *et al* (1989) neutralising the infectivity of sporozoites *in vitro*, was composed of a number of polypeptides. It was suggested that these proteins are derived from a single gene product by proteolytic processing (Williamson, 1988). The sequencing of this sporozoite gene showed that some sequences have close homology to a repetitive domain in bovine elastin (Hall *et al*, 1992). The authors suggested that this gene codes for a protein which might be a ligand binding to the elastin receptors on bovine target cells by a mimicry process. Receptors to elastin are present on a large range of cell types, including monocytes which are thought to be target cells in *T.annulata* infection (Hall *et al*, 1992). This protein would be of interest in the design of a multi-component subunit vaccine for *T.annulata*. Studies on the merozoite stage in *T.annulata* (Dickson,



1993; Dickson and Shiels, 1993) characterised a major merozoite surface antigen which is strongly recognised by immune sera. This molecule is also present in piroplasms. The gene coding for this protein has been cloned (B.R.Shiels, unpublished data) and has the potential for inclusion in a subunit vaccine.

#### 1.2.8.2 Cellular response

While the humoral response in *Theileria*-infected cattle was shown to be important against the sporozoite, the cellular response has been shown to be mainly directed against the schizont-infected cells. Early studies led to the belief that immunity directed against schizont-infected cells plays a significant role in protecting cattle exposed to theileriosis. Sergent *et al* (1939) showed that cattle could be immunised against *T.annulata* by the inoculation of blood from recovering animals (containing schizont-infected cells). The same team showed that isolates maintained in the laboratory by serial passage through susceptible calves eventually lose the capacity for the schizonts to develop into piroplasms (Sergent *et al*, 1932). The same phenomenon was reported for the "Tova" strain which was fully virulent after losing its ability to produce piroplasms (Hulliger, 1965). Such isolates were able to stimulate protective immunity which implies that the immunity was induced by the schizont-infected cells. This method of vaccination against *T.annulata*, based on the mild strain Kouba was used on large numbers of animals annually in both Algeria and Palestine (Sergent *et al*, 1945; Alder, 1952). These results led subsequent workers to consider that the mechanisms responsible for protective immunity in theileriosis were cell-mediated and targeted at the schizont-infected cells. Emery (1981) confirmed this hypothesis for *T.parva* by showing that protection can be transferred between chimaeric twins by the transfer of lymphocytes from the immune animal to the susceptible twin.

Studies on the cell mediated immune mechanisms in *Theileria* infections have been reviewed by Morrison *et al* (1986) for *T.parva* and by Hall (1988) for *T.annulata*. Significant progress in understanding the mechanisms was made subsequent to the development of a technique for the *in vitro* infection of naive PBM with sporozoites by Brown *et al* (1973) allowing the generation of autologous target cells *in vitro*. Pearson *et al* (1979) showed that

*T.parva* transformed cell lines induce a mixed leucocyte reaction (MLR) *in vitro* with autologous PBM from either naive or immune cattle. However, the infected cells were killed only when PBM from immune animals were used as effectors. Following this, several workers investigated this cytolytic activity. It was found that PBM from animals undergoing lethal infection showed cytolytic activity which was non-specific, whereas PBM from animals undergoing primary infection but which are going to recover, or challenged immune animals, contain cytotoxic cells whose killing activity is restricted to the autologous parasitised cells (Emery *et al*, 1981a; Eugui and Emery, 1981). These genetically restricted cytotoxic cells would kill autologous infected but not allogeneic infected cells. The effector cells responsible were demonstrated to be a subpopulation of T-cells (Emery *et al*, 1981b) and their phenotype in the bovine has been identified as CD2 + CD4 - CD8+ (Goddeeris *et al*, 1986). Morrison *et al* (1986) have also shown that this cytotoxic activity was MHC class I restricted. In other host-pathogen systems, the basis of recognition operating when MHC restricted cytotoxic activity is observed has been attributed to the presentation of processed antigenic peptides in intimate association with the MHC class I molecules. (Townsend *et al*, 1986).

The first investigation of *T.annulata* cellular immunity was made by Preston and Brown (1981). They showed that irradiated *T.annulata* infected cells induced proliferation in autologous PBM whether or not the cells came from an animal which was immune. Preston *et al* (1983) later reported that there were two peaks of cytotoxic activity during primary *T.annulata* infection. The first, two weeks after the contact with the parasite, was restricted to autologous infected cells, whereas the second appearing approximately four weeks after infection was not genetically restricted. The latter was associated with the recovery of the animal, since it was not detected during lethal infections. The same kinetics of response occurred during challenge of immune cattle, only both peaks appeared to be genetically restricted. It was suggested that the non-restricted activity could be due to natural killer (NK) cells, which are known to show no genetic restriction (Hall, 1988).

In a more recent analysis, Innes (1988) studied the development and specificity of cytotoxic T-lymphocytes (CTL) in three groups of animals immunised with *T.annulata*

sporozoites, or autologous or allogenic *T.annulata* infected cells. All groups developed a CTL response which was genetically restricted three weeks after the infection. However, early in the infection, animals receiving sporozoites or autologous cells showed a severe clinical reaction and a CTL response which did not appear to be genetically restricted. In contrast a mild clinical reaction occurred in recipients of allogeneic cells in association with a strong CTL response directed against the allogeneic MHC antigens simulating a graft rejection response. When the animals were challenged with sporozoites, all groups developed a CTL response 10 days later which was both parasite specific and MHC restricted. A CTL line generated from one of the sporozoite recipients was shown to kill only autologous infected cells or parasitised cells which were matched with the effector cells for MHC class I antigens. This cytotoxic activity was inhibited by MAbs to MHC class I molecules but not to MHC class II molecules, confirming that the cytotoxic cells were restricted in their function by MHC class I molecules. Phenotypic analysis showed that the effector function was due to a subpopulation of T-cells (BoT4-).

These CTL responses in both *T.parva* and *T.annulata* are transient. Therefore in order to get a constant supply of material *in vitro*, systems were developed, based on the MLR in the presence of parasite infected cells, to investigate the CTL response induced by *Theileria*. Emery and Kar (1983) showed that the cytotoxic cells, generated *in vitro* using PBM from immune or naive cattle in autologous MLR, did not exhibit genetic restriction and would target both infected and non-infected cells. The authors therefore suggested that the antigens recognised by the CTL *in vitro* are different from those recognised *in vivo* since, in the same study, CTL generated *in vivo* killed only autologous or MHC matched infected cell lines.

Despite the controversy on the specificity of CTL generated *in vitro*, CTL lines and clones specific for *T.parva* infected cells, MHC class I restricted and even parasite strain specific, have been generated (Goddeeris *et al*, 1986; Taracha *et al*, 1991). T-cell lines are an important tool for the identification of the antigen(s) recognised by the CTL which could be infection specific antigens, possibly of parasite origin, on the surface of the schizont-infected cells. These molecules would represent ideal candidates for a subunit vaccine. With the idea

of identifying these molecules, attempts have been made to select MAbs reacting specifically with the surface of the infected cells. The only success to date for *T.annulata* has been obtained by Shiels *et al* (1986b). The authors demonstrated that one MAb (4H5) reacted specifically with the surface of *T.annulata* infected cells and, in addition, mediated a complement lysis of such cells (Preston *et al*, 1986), showing that the identified epitope can be the target of the immune response. However it is still not clear whether the molecules recognised are encoded by the host cell or by the parasite.

The cellular immune response to sporozoites, merozoites and piroplasms has not been fully investigated, possibly because of the limited role that these stages play in inducing cellular immunity and the difficulty in obtaining pure viable parasite material from them.

### **1.2.9 Control methods**

#### **1.2.9.1 Tick control**

Farm management and vector tick control play significant roles in reducing the impact of theileriosis in endemic areas. Fewer ticks, imply fewer sporozoites, therefore milder disease and a greater chance of recovery for the infected animals (Gill *et al*, 1981).

The control is aimed at limiting the contact between the animals and the vector tick. *Hyalomma* spp are cowshed dwelling ticks. Thus, improvement of the construction of stables and removal of sites in which hibernating ticks can hide would certainly decrease the impact of the disease. Additionally, the regular use of acaricide on animals and cowsheds, especially during the peak of *Hyalomma* activity, should diminish the incidence of theileriosis without interfering with the enzootic stability. (This is discussed in relation to Tunisia in section 1.3.3).

#### **1.2.9.2 Chemotherapy**

In areas where a vaccine against tropical theileriosis is not yet available, such as Tunisia, the control of the disease is based on acaricide use and chemotherapy. Early studies reported a number of drugs have been tested and some of them were reported to have an anti-theilerial activity, however none of these was entirely reliable (Neitz, 1957).

Tetracyclines (Neitz, 1953) have also been used but have proved less satisfactory than the therapeutics currently used. They were shown to be more effective in the infection and treatment methods to limit the clinical disease during immunisation with sporozoites than to cure animals once the disease has been established (Neitz, 1957). The three currently used drugs are halofuginone (Schein and Voigt, 1979), parvaquone (McHardy *et al*, 1976) and more recently buparvaquone (McHardy *et al*, 1985). Halofuginone although effective against the clinical disease, was toxic for the animals at doses close to the therapeutic dose used (Schein and Voigt, 1981). The naphtoquinones are both analogues of the menoctone (Dolan and McHardy, 1978). They specifically kill the intralymphocytic schizonts but in the case of buparvaquone, are also effective against the intraerythrocytic piroplasms (McHardy *et al*, 1983; McHardy and Wekesa, 1985). These drugs are safe and very effective against the disease but are still very expensive (Brown, 1990) and must be administered early on in the course of the clinical attack to be effective.

#### **1.2.9.3 Resistant breeds**

The use of breeds resistant to both theileriosis and the tick vector, could play a major role in the control of the disease. However, studies on genetic resistance to theileriosis in cattle have yet to be conducted. First approaches would indicate that many resistant breeds, which, generally are simply indigenous cattle, are poorly productive (Spooner and Brown, 1991). However, if the genes determining resistance could be identified, the possibility of introducing these into highly productive cattle is a possibility.

#### **1.2.9.4 Immunisation**

Two methods have been used so far to immunise cattle against theileriosis, the infection and treatment method and the inoculation of attenuated schizont-infected cell lines.

Infection of animals with sporozoites and simultaneous treatment with tetracyclines is the method currently used to immunise cattle against *T.parva* (Norval *et al*, 1992) but has also been shown to be effective against *T.annulata* (Gill *et al*, 1976).

Cell line vaccines effective against tropical theileriosis have been used in many

countries and give good protection in areas where the disease is endemic (Pipano, 1990). The duration and mechanisms of the immunity provided by such vaccines however, have not yet been fully investigated.

Sergent *et al* (1932) tested several isolates passaged mechanically between cattle with the aim of detecting a naturally avirulent strain. They found natural differences in virulence between strains isolated from different sites in Algeria. From these they used several relatively mild strains to vaccinate cattle. They also found that the virulence of a strain does not vary when it is transmitted from cow to cow and there is neither natural attenuation nor natural exaltation of virulence of strains by serial passages in cows. However Rafyi *et al* (1965) stated the opposite. These authors discussed the difficulty in maintaining the virulence of a strain constant *in vivo* because *T.annulata* strains lose their virulence gradually during serial passage in cattle.

Sergent *et al* (1939) used small quantity of infected blood (5-10mls) for vaccination, administered subcutaneously. Their results were not consistent and there was a relatively high risk of mortality in the vaccinated animals. They established that by using the mildest of the strains they had isolated, the Kouba strain, the rate of mortality in inoculated calves was 3%. This strain was maintained for 11 and half years, by serial passage in cattle, and was used as the vaccine strain during this time. It was lost at its 223rd passage as were two other mild but more virulent strains used extensively, the "St.Charles" and the "Brunette" strains (Rafyi *et al*, 1965). Sergent *et al* (1932) reported that the Kouba strain failed to produce piroplasms after the 18th passage. Such mild strains were used in Algeria, Morocco and Tunisia for 19 years to vaccinate more than 36,000 animals. The mortality rate in vaccinated animals exposed to natural challenge by the parasite in the field was reported to be only 1% as opposed to 16.3% in non-vaccinated exposed animals. The losses due to the vaccination itself were estimated to be 0.8% of the vaccinated animals (Anonymous, 1949). In Palestine, Adler and Ellenbogen (1934) were unable to isolate an indigenous *T.annulata* strain with low virulence, so they acquired the Kouba strain from Algeria. The authors reported that the strain gave poor protection against local virulent strains and adopted a two-step procedure. First they inoculated the Kouba strain, followed two months later by schizont-infected cells



of a local strain to reinforce the immunity. It is believed that they immunised approximately 260,000 animals between 1933 and 1950 (Adler, 1952). Similarly in Iran, in 1939, 48 cows were imported from France where they had been vaccinated with the Kouba strain. Four of them died from theileriosis, after their arrival in Iran (Hooshmand-Rad, 1973). Nevertheless this form of immunisation has made importation of improved cattle possible in these endemic areas and in addition made the way to show the vaccine.

The duration of immunity engendered by this blood inoculation was estimated to be one year in the absence of natural challenge (Sergent *et al*, 1945). Tsur *et al* (1964b) reported an outbreak of theileriosis with mortality in animals which had been vaccinated three to four years previously, indicating presumably that the animals were no longer protected after such a long period of time. They suggested that vaccination should be repeated every year.

The use of a blood vaccine has many disadvantages. Apart from the risk of causing death, it has to be maintained by serial passage through cattle, and the risk of transmission of other pathogens from an animal with intercurrent infection is a concern. This occurred with the Australian *Babesia* vaccine spreading the bovine leukosis virus (Rogers *et al*, 1988).

Major steps towards another means of vaccination were made when Tsur (1945) first cultivated *T.annulata* schizont infected cells in plasma clot cultures. This was consolidated later, when Tsur *et al* (1964a) achieved the mass propagation of the parasite in tissue culture. It was not until the studies of Hulliger *et al* (1964) that the mode of multiplication of *Theileria* in lymphoid cells was elucidated (see Section 1.2.6). In 1965, Tsur, suggested the possibility of using tissue cultured *T.annulata*-infected cells to provide material for vaccination (Tsur, 1965). The phenomenon of attenuation, reduced virulence, of the parasitic after prolonged cultivation *in vitro* was first reported by Tsur and Pipano (1966). The mechanisms of this attenuation are not fully understood. *T.annulata*-infected cell lines when attenuated generally conserve their immunogenicity, whereas with *T.parva*-infected cell lines the attenuation is accompanied by loss of immunogenicity (Brown, 1981).

Tsur and Pipano (1966) showed that *T.annulata* cell lines cultured over long periods, with many passages *in vitro*, reached a point where no temperature response or parasites

were detectable when they were inoculated into animals. The authors claimed that all animals receiving such cell lines were immune to subsequent challenge with a virulent strain of schizont-infected cells which caused 30% mortality in non-immunised animals.

The development of methods of cryopreservation for *T.annulata* was also a major step towards making vaccination feasible. Tsur and Pipano (1962) demonstrated that the schizont-infected cells could be frozen at -70°C in 15% glycerol and stay viable for at least 150 days. Currently schizont vaccines can be cryopreserved, resuscitated, cultured for a short term and used as a fresh suspension. However in some cases vaccines are cryopreserved in liquid nitrogen and used directly without being cultured (Pipano, 1989).

The protection engendered by the tissue culture vaccine does not necessarily relate directly to the number of passages *in vitro* undergone by the cell line or to whether or not the vaccine cell line is producing piroplasms. Hooshmand-Rad (1973) showed that animals immunised with a strain A, passaged for 290 days in tissue culture and not producing erythrocytic forms, gave full protection against a virulent heterologous challenge. This was in contrast to a strain B, passaged for only 45 days in tissue culture and producing piroplasms, which only protected two out of six vaccinated animals against the same virulent challenge.

*T.annulata* cell culture vaccine methods were further developed by Pipano (1981) and have subsequently been used in several countries (Ouhelli, 1991; Zablotsky, 1991; Hashemi-Fesharki, 1988; Sayin, 1991; Singh, 1991). The number of cells per vaccine dose varies between  $10^4$  and  $10^7$  cells, with the number of days in culture, when known, ranging from 120-912 days. The duration of immunity engendered by those cell culture vaccines is also variable and ranges from 6 months (Ouhelli, 1991) to 3.5 years (Zablotsky, 1991). However the duration of immunity produced by some of the vaccines has only been tested for up to 6 months (Ouhelli, 1991). Given this variability, it is very difficult to compare data from different countries concerning the efficiency and safety of cell culture vaccines and the level of protection provided. Zablotsky (1991) reported that piroplasms could be detected at fluctuating levels in blood smears seven months after vaccination. During these periods the author intimated that there was a possibility that vector ticks feeding on vaccinated animals could transmit the parasite to susceptible cattle.



It is generally believed that tissue culture vaccines may represent a risk to lactating or pregnant cows, due to the febrile reaction which can occur post vaccination. (Pipano, 1977; Hashemi-Fesharki, 1988).

The process by which the schizonts are transferred from the donor cells to the recipient animal cells has yet to be elucidated but may involve phagocytosis, entry of free parasites liberated from lysed inoculated cells into recipient cells or cell fusion, shown to take place by Irvin *et al* (1974). Innes (1988) investigated the effect of histocompatibility between the cells of the vaccine cell line and those of the recipient. Susceptible cattle were successfully infected and immunised with *T.annulata*-infected cell lines at doses ranging from  $10^2$ - $10^8$ , whether the animals were MHC matched or mismatched to the donor cell line. In contrast Brown *et al* (1978) and Dolan *et al* (1984) showed that there was a histocompatibility barrier for cattle that received MHC mismatched *T.parva* infected cells. Morrison *et al* (1981) demonstrated that infection could be induced with as few as  $10^2$  autologous *T.parva*-infected cells, whereas  $10^8$  cells were needed in mismatched animals, at which dose, the risks of lethal infection were high. These findings would partly explain why a tissue culture vaccine in *T.parva* has not been developed and may relate to the different phenotype of cells infected by *T.annulata* and *T.parva* (Innes *et al*, 1992).

As a result, another method, known as the infection and treatment method of immunisation was developed and is used to immunise against East Coast fever. The immunisation procedure is based on the inoculation of a controlled dose of *T.parva* sporozoites at the same time as a chemotherapeutic drug, usually long-acting oxytetracyclines. Sporozoites alone do not provide a reliable means of immunisation, as there is considerable variation in the response in groups of cattle receiving doses approaching the LD50, from no infection to death, with all intermediate states of the disease (Norval *et al*, 1992). The simultaneous treatment with oxytetracyclines and infection with sporozoites, results in an effective method of immunisation because it allows the parasite to be established but not at a level to produce disease (Radley, 1981). However this method, although the only one presently available for *T.parva*, has many disadvantages. Firstly it is relatively expensive, compared to the *T.annulata* cell line vaccine (Mukhebi *et al*, 1992). Secondly, post

immunisation breakthroughs occasionally occur despite the chemoprophylactic cover, resulting in disease which must be treated. Thirdly the protection provided against other stocks by a given *T.parva* stock is variable in both laboratory conditions and under natural tick challenge in the field (Norval *et al*, 1992). Therefore other methods of immunisation are being investigated to provide safer and wider protection against East Coast fever.

The fact that CTL kill the parasite-infected cell and are a major defence against the disease, has led workers to attempt the development of novel vaccination strategies based on the identification of parasite antigens recognised by the CTL. Such antigens would provide good candidates for recombinant vaccines against *T.parva*. However, a major difficulty is presented by the MHC phenotypes which influence the CTL responses in theileriosis. Nevertheless, and in other systems, using the p67 antigen of *T.parva* a recombinant form of a protein on the surface of the sporozoite (Musoke *et al*, 1992), it has been shown that several non-related MHC-heterozygous cattle that differed in class I MHC phenotype responded similarly to immunisation with this antigen, suggesting that MHC phenotypes do not always interfere with responses of cattle to protein antigens (Anonymous, 1991).

Hill *et al* (1992) showed a statistical association between HLA-B53 class I antigen and resistance to severe malaria. They also showed that HLA-B53 positive cells can bind a peptide from the liver-stage-specific-antigen-1 (LSA-1) and that LSA-1 specific CTL are present in the circulation of some malaria immune people. The identified antigen from LSA-1 (1s6) might represent a component of a vaccine which will induce a CTL response and confer some immunity against the malaria parasite (Cox, 1992).

Thus after several years, the search for a vaccine in both malaria and East Coast fever is moving away from the antibody-mediated responses to the sporozoites, to the CTL-mediated responses to antigens expressed on the surface of infected-cells. The identification

of *Theileria* epitopes that provoke the CTL responses will be decisive in the design of a molecular recombinant vaccine. Whether or not these protective epitopes contain polymorphic residues, is still to be investigated and studies exploring the diversity of the parasite might provide a useful background for analysis of the extent of variability in such epitopes using well-defined and characterised cloned parasite populations.

Although a recombinant vaccine for tropical theileriosis is not as desperately needed as it is for East Coast fever, this type of research, applied to *T. annulata*, would help in avoiding specific problems related to live attenuated cell line vaccines such as the storage and delivery of the vaccine, its safety, cost-effectiveness and potential for reservoir to virulence.

#### **1.2.10 Pathogenesis**

The main symptoms observed in tropical theileriosis are fever (up to 41 or 42°C) accompanied often with loss of appetite and rumination and an enlargement of the external lymph nodes (prescapular and precrural in particular). The mucosa are congested and petechiae appear. Lactation is arrested in females and some abort or give birth prematurely (Sergent *et al*, 1945). Late in the disease, there is blanching of mucosal membranes as anaemia becomes evident.

The pathogenesis of the disease is due to two major mechanisms, the lymphoproliferation and subsequent destruction of the infected lymphoid cells, and the anaemia. The disease, in experimentally infected animals, starts with an enlargement of the lymph node draining the site of sporozoite inoculation, followed by a marked leucopenia and a later fall in the packed cell volume due to the anaemia. Two peaks of temperature are often observed in these animals. The first, 7-8 days after the inoculation, correlates with the detection of the schizonts in the lymph node draining the site of inoculation, whereas the second peak, appears around the second week of infection and seems to be synchronous with the appearance of the piroplasm in blood smears. The anaemia observed in acute and subacute forms of the disease does not always relate to the percentage of parasitised cells, and might imply other mechanisms than the destruction of the red blood cells, such as auto-immune reactions (Hooshmand-Rad, 1976). The disease can be fatal due to the severe

panleucopenia, anaemia and liver failure. Death occurs around 20 days in susceptible animals. In animals which succumb to tropical theileriosis, several internal organs are found to be infiltrated with lymphoblastoid cells notably liver, spleen, lungs, kidneys, adrenals, lymph nodes and some areas of the gut (Sergent *et al*, 1945).

Low levels of parasitaemia are detected in recovered animals which become carriers of the disease and are a source of infection for the vector tick. The persistence of the parasite in recovered animals is responsible for the state of premunition which interferes with the development of further clinical disease (Sergent *et al*, 1945).

### 1.3. EPIDEMIOLOGY OF THEILERIOSIS IN TUNISIA

The disease is primarily confined in areas where the vector tick is present. In Tunisia, one third of the country is disease free. The epidemiology is determined by the tick distribution and its seasonal activity.

Early studies conducted at the Institut Arloin in Tunisia have emphasised the role of piroplasmoses and their respective tick vectors in the economic losses registered in the cattle population (Cordier, 1941). These comprised the two babesiosis caused by *B. bigemina* and *B. bovis*, anaplasmosis, caused by *Anaplasma marginale* and theileriosis, caused by *T. annulata*. The latter being the most devastating disease (Cordier and Menager, 1940). The mortality due to theileriosis was around 50% in susceptible cattle but mortality rates as high as 100% were recorded in some stables (Cordier, 1941). The disease was described as enzootic with percentages of mortality and morbidity as high as those recorded in important epizooties (Cordier, 1941). Experiments conducted at the Institut Arloin on Zebus (Nellore and Scindh) have shown that they were less susceptible to tropical theileriosis than *Bos taurus* cattle (Cordier and Menager, 1940). In contrast studies conducted in Morocco have shown the full susceptibility of these animals when newly introduced to endemic areas (Gayot, 1953). The susceptibility to the disease was also shown to be dependent on the age of the animal, the young calves being more resistant than the adults, and the type of production, the dairy cows representing the most susceptible category (Cordier and Menager, 1940). In the same study the authors reported the importance of the geographical

location of the stables. Those at high altitudes were less affected than those in the plains.

The Kouba strain was sent regularly from the Institut Pasteur d'Algerie (IPA) to vaccinate cattle in Tunisia. Vaccination was conducted for 8 years (1931-1939) and in the immunised animals, 4.62% morbidity and 1.54% mortality were recorded compared to 9.09% mortality in control groups in the same farms (Cordier *et al*, 1936; Cordier and Menager, 1940).

Vaccination was interrupted after trials, in 1939, showed important vaccinal reactions. On investigation, the workers at the Institut Arloin discovered that the strain sent by the IPA in 1939 was the "Brunette" strain. The Kouba strain was lost and no longer available. The Brunette strain although more virulent than the Kouba strain, did not give any significant vaccinal reactions in immunised animals at the IPA. However, when used in Tunisia, it not only caused mortality in vaccinated animals but also, since this strain was still producing piroplasms (the Kouba strain did not produce any), was responsible for the appearance of theileriosis in farms, in the summer following the vaccination, where the disease had not been recorded before, (Cordier and Menager, 1940).

It is worth noting that a study by Gayot (1953) analysing the important threats to the cattle population in Tunisia, suggested the use of culture of the parasite from infected tissues (spleen, liver, lymph node) in order to supply a continuous source to conduct studies on *Theileria* and theilericidal drugs. The author was concerned about the cost of such research and suggested that, since it is a world wide problem several countries such as Morocco, Algeria, Egypt, countries of the Middle East, Turkey, Greece and Iran, should get together and set up an international project to fight the disease with the help of international agencies such as FAO.

It was not until 1970 that further studies on theileriosis were again in focus. Indeed Van den Ende (1970) while conducting a study on the important species of *Ixodidae* ticks in Tunisia and their repartition, showed that *H.detrutum* was the most frequent species found on cattle. The same study argued the role of *H.a.excavatum* in transmitting tropical theileriosis in arid regions. This tick was also found on sheep and hibernates in the fields in these regions (Fourgi and Sornicle, 1962). *H.a.excavatum* is far more frequent in arid areas compared to

*H.detrutum* and therefore might play a role in the transmission of *T.annulata*. However *H.a.excavatum* instars usually feed on small rodents especially *Meriones shawi* (Van den Ende, 1970). In Tunisia Van den Ende and Edlinger (1971), were the first to culture *T.annulata*-infected cell lines in suspension and using for the first time RPMI medium. However no further studies were conducted at this time and it is only in 1988 that a project aimed at producing a vaccine was conducted by Dr M. Darghouth (ENMV) in collaboration with Dr A.Bouattour (IPT). Concurrent epidemiological studies were also planned on the vertebrate and invertebrate host. Most of the data presented, in the following sections, are based on the few studies carried out recently, some of which are still underway. These studies concern the distribution of the disease in cattle, the biology, distribution and evaluation of the infection rate in the tick vector, the disease stability, its importance and economic impact and the control measures applied.

### **1.3.1 Distribution of the disease in the cattle population**

A study by the Ministry of Agriculture, in 1991 showed that 30% of the breeding cows are of improved European breeds and that 80% of the theileriosis cases were registered as occurring in cattle of these breeds. The total cattle population in Tunisia was estimated as 631, 180 head in the same study. Their distribution is shown in Table 1.2. It is seen that the majority (81%) of the cattle are located in the North of the country, therefore it is not surprising that most (85%) of the theileriosis cases were recorded from the northern "Gouvernerats" (administrative regions, Fig. 1.4) of the country. Around 2500 cases occur each year. In 1991, 2140 cases were recorded, the proportions registered in each gouvernerat are shown in Table 1.2.

### **1.3.2 Distribution, biology and *T.annulata* infection rate of the vector tick: *H.detrutum***

In Tunisia the most important species transmitting *T.annulata* in the field is *H.detrutum* (Bouattour *et al*, 1993). Sargent *et al* (1945) reported that *H.detrutum* (*H.mauritanicum*) was the principal vector of tropical theileriosis in Algeria, as did Flach and Ouhelli (1992) in Morocco.

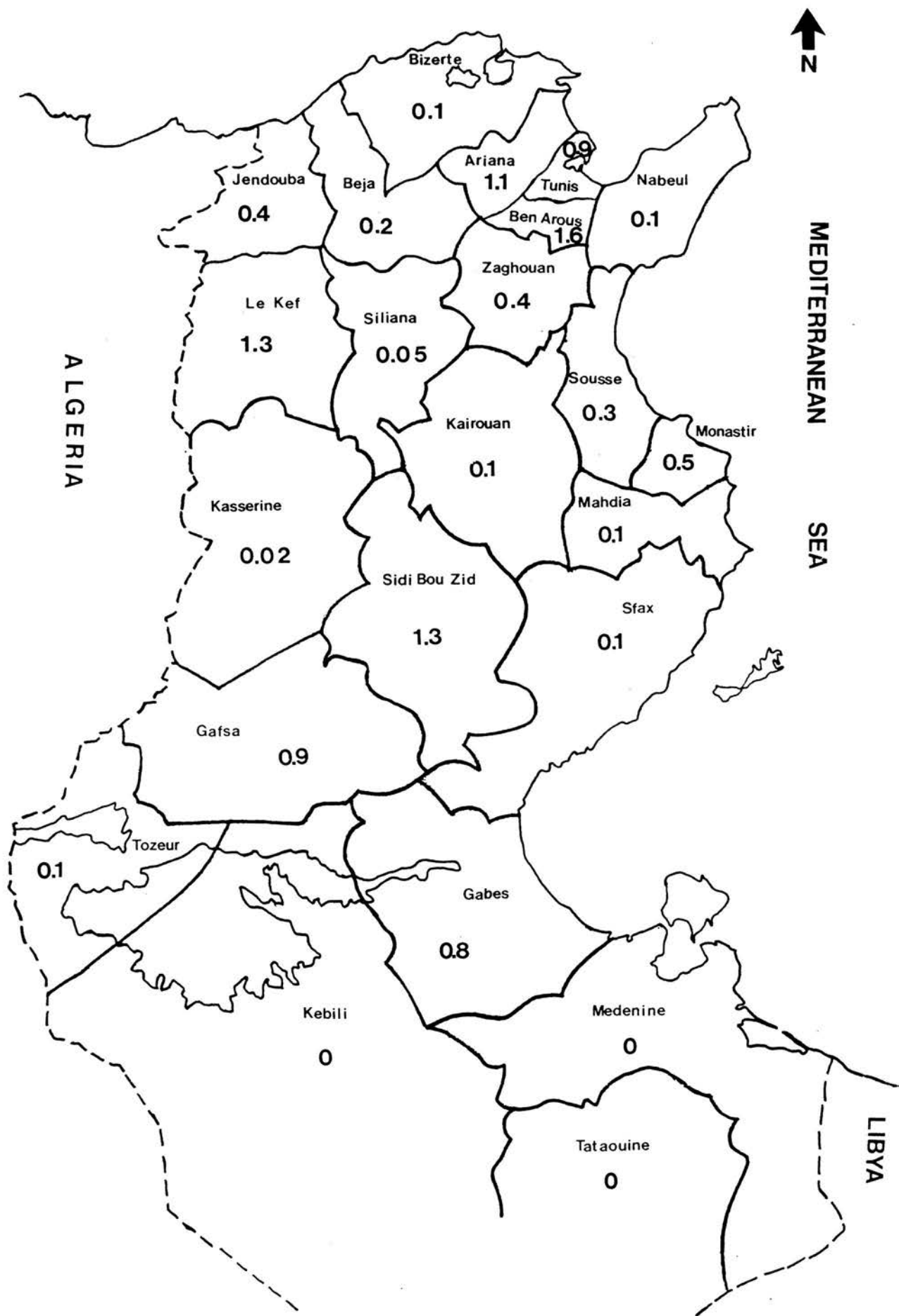


**Table 1.2** Bovine population distribution and theileriosis cases registered in 1991  
(Ministry of Agriculture - TUNISIA).

Gouvernerats	Bovine population		Theileriosis cases		Disease incidence
<b>NORTH</b>	<b>513920</b>	<b>81.8%</b>	<b>1821</b>	<b>85.0%</b>	
Tunis	4960	0.7%	42	2.0%	0.9%
Ariana	39140	6.2%	419	19.6%	1.1%
Ben Arous	9000	1.4%	150	7.0%	1.6%
Nabeul	74970	11.9%	50	2.3%	0.1%
Bizerte	110000	17.4%	93	4.3%	0.1%
Beja	94130	14.9%	183	8.5%	0.2%
Jendouba	81700	12.9%	316	14.8%	0.4%
Le Kef	36640	5.8%	478	22.3%	1.3%
Siliana	49220	7.8%	27	1.3%	0.05%
Zaghouan	14430	2.3%	63	2.9%	0.4%
<b>CENTRE</b>	<b>110510</b>	<b>17.5%</b>	<b>296</b>	<b>13.8%</b>	
Sousse	11170	1.8%	34	1.6%	0.3%
Monastir	9160	1.4%	50	2.3%	0.5%
Mahdia	7020	1.1%	6	0.3%	0.1%
Sfax	12100	1.9%	10	0.5%	0.1%
Kairouan	49160	7.8%	71	3.3%	0.1%
Kasserine	11900	1.9%	2	0.1%	0.02%
Sidi Bouzid	7000	1.1%	95	4.4%	1.3%
Gafsa	3000	0.5%	28	1.3%	0.9%
<b>SOUTH</b>	<b>6750</b>	<b>1.1%</b>	<b>23</b>	<b>1.1%</b>	
Gabes	2450	0.4%	21	1.0%	0.8%
Mednine	850	0.1%	0	0%	0%
Tozeur	3250	0.5%	2	0.1%	0.1%
Kebili	100	0.01%	0	0%	0%
Tataouine	100	0.01%	0	0%	0%
<b>Total</b>	<b>631180</b>		<b>2140</b>		<b>0.3%</b>

**Figure 1.4** Map of Tunisia showing the disease incidence in the different administrative regions of the country (Gouvernerats).





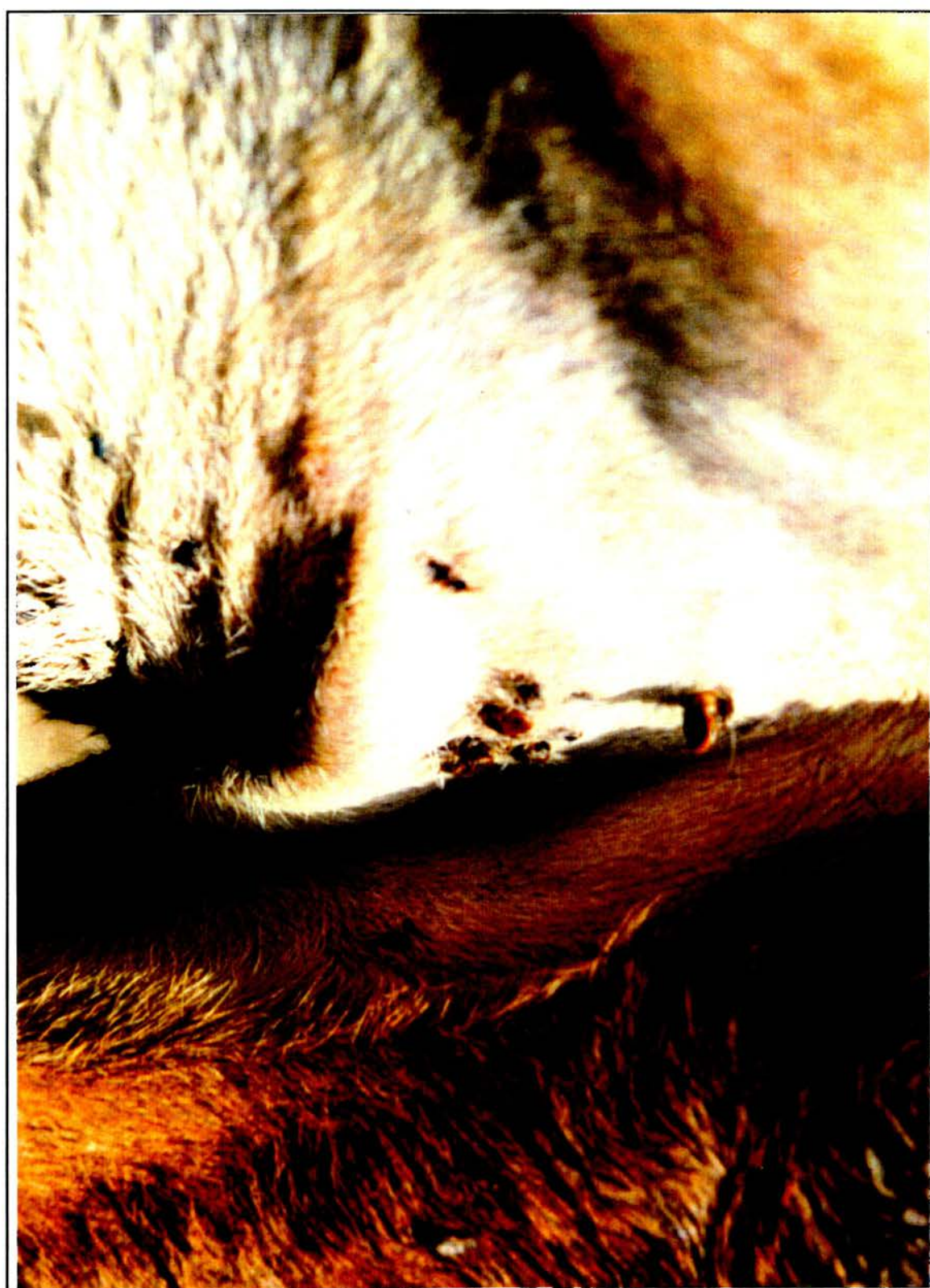
*H. detritum* is found in areas characterised by marshes, riparian flood plains, irrigated fields and grassy slopes of desert and semidesert areas. The larvae of this two-host tick hatch from eggs which are laid on the ground and several days later they begin to seek a host on which to attach and engorge before moulting to nymphs (September-October, Fig. 1.2, Section 1.2.6). The nymphs in turn take a blood meal from the same host. The engorged nymphs drop to the ground to moult around April-May (Fig. 1.2). Males and females emerge from this moult and search for a new host. They attach, often in the anal, perineal and vulval regions or on the inside of the hind legs, hidden by the udder. Fertilisation takes place on the host (Fig. 1.5) and the female then completes a large blood meal and drops to the ground five to seven days after attaching. She lays approximately 4,000 eggs (Figs. 1.2 and 1.6).

The immature and adult instars normally feed on domestic cattle. The moulting nymphs are usually found in crevices and cracks of walls under rocks, and even under dried manure (Ben Miled *et al*, 1993). Very often the ticks appear to choose sites within walls which are exposed to the sun, to hibernate. *H. detritum* is a stable dwelling tick but can be found in the areas immediately surrounding the cowshed. The adults, when looking for a host can move 20-30 metres (Bouattour, 1982).

The tick is only active during a short period of the year in Tunisia: in the summer season. The adult activity starts in April-May and finishes towards the end of September (Fig. 1.2). The females feed for approximately 10 days and drop whereas the males remain on the animals sometimes up to 60 days. Infestation of livestock by immature instars occurs at the end of summer and beginning of autumn (Fig. 1.2). The immature instars remain attached to their host for three to four weeks. Then the engorged nymphs detach and hibernate from November until the temperatures rise at the beginning of spring.

Darghouth (1992) studied the proportion of *H. detritum* females among ticks collected on domestic cattle between June and August 1991. The peak of female activity correlated with the peak of theileriosis cases recorded by the Parasitology Laboratory, Ecole Nationale de Medecine Veterinaire (ENMV), Sidi Thabet, through examination of Giemsa stained blood smears from domestic cattle for the diagnosis of theileriosis.

**Figure 1.5** Male and female ticks of *H.detritum* mating and engorging on an adult co  
Tunisia



**Figure 1.6** *Hyalomma detritum* female laying eggs





A study by Bouattour *et al* (1993) on 5,077 ticks collected from domestic cattle, on regular visits to the same farms, over one year (June 1991-June 1992), showed that 50% of the ticks collected were of the genus *Hyalomma* and that 85% of these were of *H.detrutum*. The remaining *Hyalomma* ticks were represented by *H.marginatum* (12%) and *H.a.excavatum* (3%). In the same study, the authors evaluated the infection rate of *T.annulata* infection in *H.detrutum* ticks using methyl green pyronin (MGP) staining of the salivary glands (Walker *et al*, 1979). They found that around 13% of the ticks dissected contained typical *T.annulata* infected acini. Only females of the other *Hyalomma* species were dissected and they did not show any sign of infection with *T.annulata*, confirming that *H.detrutum* is far and away the most important and perhaps the only significant vector for the disease in Tunisia.

In a similar study in Morocco, Flach and Ouhelli (1992) showed that the principal vector of *T.annulata* was *H.detrutum* and that the infection rate calculated from the examination of 127 ticks collected over the summer of 1990 was 19%.

### 1.3.3 Disease stability and instability.

An epidemiological study on two types of farms, A and B, located in two areas close to the ENMV, was carried out by Darghouth *et al* (1992). Animals on farms of type A were exposed to moderate levels of tick infestation whereas animals on farms of type B were exposed to high levels of tick infestation. As many as 200 ticks were found on animals living on type B farms. Farms of type A were situated in a relatively dry area with numerous hills while farms of type B were near the sea in an area where large marshes are present during the rainy seasons. The sampling method to determine the incidence and prevalence of theileriosis in these farms was mainly based on serology. Approximately 400 animals were monitored by IFAT (using schizont-infected cell antigens) before the theileriosis season (May 1991), after the peak season (September 1991) and before the following disease season (April, 1992). The animals were divided into four age groups according to the number of theileriosis seasons they had been exposed to. The results showed different epidemiological situations in farms from type A and type B. In both farm types the prevalence of the disease increased

with the increase in age and was similar before the first and second disease seasons in each farm type (May 1991 and April 1992).

The percentage of positive sera before the theileriosis season for animals on type A farms varied from approximately 5% (animals <7 months) to 35% (animals >2.5 years) with a mean for all age groups of 19%. After the disease season these percentages increased respectively to 9% and 45% with a mean of 30%. Animals on type B farms exhibited a different pattern. The number of positive sera from these animals varied from 30% to 95% in the corresponding age groups before the disease season, with a mean of 50%, rising to 60 to 95% after the disease season with a mean of 85% for all age groups. From these results the authors concluded that two factors influence the epidemiology of theileriosis, the age of the animals and the level of tick exposure. The number of positive animals increased with the number of disease seasons through which the animals had passed. This means that the older the animals become the higher is the probability of being subjected to tick infestation.

In the same study (Darghouth *et al*, 1992) it was noticed that clinical cases on type A farms during the summer of 1991 were only seen in animals of more than 7 months of age, (Fig. 1.7), with an incidence of the disease in all age groups of approximately 2%. These results indicated that there was endemic instability for tropical theileriosis in type A farms, because a proportion of the population remained unexposed for one or more disease season, and when they become infected for the first time disease often occurred in these animals. The situation in the second type of farm was different; nearly every animal became infected in calfhood. The animals were exposed to ticks early in life, therefore the epidemiology of the disease was stable. Fewer disease cases were recorded from these farms because the organism was widespread and most of the cattle population was immune. Indeed only a very mild form of the disease was detected in one 6 month old calf, during that summer, amongst the animals living in the type B farms.

Because of the early exposure, the prevalence of the antibodies to this infection in all age groups, but especially in animals aged less than 7 months, was higher in type B farms than in type A farms.

In conclusion, two areas only 10Km apart, in the same bioclimatic region (semi arid),



**Figure 1.7** Adult brown Swiss cow photographed one month after suffering clinical theileriosis which was treated. Notice the loss of condition.



showed endemic stability on one hand, due to a high level of tick exposure, and endemic instability on the other hand. This study showed that it is important to consider local climatic variations when carrying out a survey on a large scale in a given country.

#### **1.3.4 Importance and economic impact of the disease**

Tropical theileriosis represents one of the most important threats to the cattle population in Tunisia (others include tuberculosis, foot and mouth disease). Bouattour *et al*, (1993) reported that 87.8% of bovine piroplasmosis (theileriosis, babesiosis and anaplasmosis) is due to *T.annulata*.

Table 1.2 (Section 1.3.1) shows the bovine population distribution and theileriosis cases recorded in 1991 in each gouvernerat by the Ministry of Agriculture in Tunisia. The disease incidence calculated from Table 1.2 is approximately 0.3%. The study of Darghouth *et al* (1992) showed a disease incidence of 2%. The differences noticed could be explained by the fact that animals in the survey of Darghouth *et al* (1992) were closely watched, therefore even subclinical forms were detected and treated even though unnoticed by the farmer. In addition the area covered in their study, surrounding the ENMV, is in the gouvernerat of Ariana (Fig. 1.4) which according to Table 1.2 showed one of the highest levels of disease incidence (1.1%).

The threat represented by tropical theileriosis might not seem to be very important for the indigenous cattle population, although the economic impact of the disease resulting from milk and meat losses in recovered animals has not been examined. However, it is a major problem in improved, imported European breeds, or for the movement of indigenous animals from disease free or low risk areas into endemic areas.

Nowadays the impact of the disease is reduced because of the control measures taken by the government and discussed in the next section. Bahri (1991) reported that the value of cattle cured successfully of tropical theileriosis is estimated at 2 million Dinars per year (1D = 1 \$ U.S.) (The minimum wage in Tunisia is around 150D per month). The cost of disease control in 1987 was estimated by the Ministry of Agriculture to be around 90,000D. Bahri (1991) showed that the cost of the drug used, parvaquone (Clexon, Pitman-Moore) was

around 120,000D per year. It must be kept in mind that this figure excludes the cost of acaricide, if used and the veterinary expenses.

*1.3.5 Control*

The only means of controlling tropical theileriosis in Tunisia at present are the use of acaricide and drug treatment. Both of these are provided by the government which in 1991 distributed around 2,000 litres of Diazinon to 5,000 farms covering approximately 50,000 cattle (Bahri, 1991). This programme recommends the use of acaricide on both animals and stables in the autumn, spring and summer according to the following schedule:

September-October	Animals and stables, once a fortnight.
November	Stables, once a fortnight
March	Stables, once a fortnight
April - August	Animals, once a week.

Bahri (1991) showed that this programme was followed in 15 of 20 gouvernerats, but not all farmers in the 15 gouvernerats applied the acaricide treatment on a regular basis. For example, in three gouvernerats more than 75% of the farmers given the acaricide were following the recommended programme regularly, whereas in seven other gouvernerats, less than 25% were doing so. Therefore one of the major problem in the use of acaricide is its correct application by the farmers. However, the programme suggested involves extensive application and one possibility to increase its effectiveness is to target only the immature ticks. This would mean treatment only in September, October and November. In this way, the government would cut the number of doses of acaricide used in each farm and would be able to cover a larger number of farms by the programme for the same cost.

The application of an intensive acaricide programme, as the one recommended by the Ministry of Agriculture in Tunisia, might have some consequences which could affect the stability of the disease in endemic areas. Indeed such programmes would imply that it is targeting the eradication of the disease. Such eradication is very difficult to perform since

nothing is known about the role of sheep and goats as carriers. Therefore treatments to restrict the numbers of ticks on cattle and elimination of their resting sites by improvement of the standards of housing would be more appropriate in order to maintain endemic stability. This is why targeting only the immature ticks, might be economically as well as epidemiologically suitable for Tunisia. The application of acaricide to animals in Tunisia, is mainly by hand spray which is the simplest method but potentially the least effective because it depends on the thoroughness of the operator in applying the acaricide.

Once clinical theileriosis is diagnosed by a veterinarian in Tunisia, the theilericidal drug (parvaquone/buparvaquone) is obtained free, since most of the farmers, especially small holders, can not afford the cost of the treatment (70D per dose). This policy is certainly an appropriate solution since the loss of infected animals would be far more important for the country than the total cost of the treatment provided against tropical theileriosis. The study by the Ministry of Agriculture of Tunisia showed that 1470 animals received free treatment out of 2140 suffering clinical disease. Bahri (1991) showed that in 50% of the cases recorded, the animals received two doses of theilericidal drug rather than one, and 91% of these were also provided with adjuvant treatment. Thus the cost of the drug provided by the government was approximately 150,000D and was underestimated in the study of Bahri (1991). In any case these figures are an underestimate of the real cost of the disease which includes the cost of the adjuvant treatments, the use of acaricide, the veterinary expenses and losses in milk and meat in the infected animals.

Considering the cost of existing control measures and the value of the animals treated, it is far more economic to consider the replacement of existing control programme by vaccination. An estimate of the cost of a non-frozen vaccine, based on other countries, would be approximately 0.5D a dose (C.G.D.Brown, personal communication). Therefore by targeting the populations at risk, i.e. the improved breeds (around 100,000 head) and calves of less than two years old (around 130,000) (Ministry of Agriculture, Tunisia, 1991), 115,000D would be needed per year for a vaccination programme in Tunisia. This is not even equal to the cost (120,000D) of the drug used in the control programme reported by Bahri (1991) and far more economic than the real cost of the disease. In addition the cost of such



vaccine, if produced in Tunisia, would be in local currency as compared to the foreign currency needed to purchase drugs and acaricide. For all the reasons listed above, this study in conjunction with the development of a cell culture vaccine were planned. The impact of these studies is therefore both important at the fundamental level as well as the economic level. This type of research is a priority in developing countries, such as Tunisia.

The development of a cell culture vaccine is underway at the ENMV. Four schizont-infected cell lines have been selected, based on the geographical locations of their origin and the polymorphism shown by both phenotypic and genomic markers (Ben Miled *et al*, 1991). These cell lines have been attenuated by Dr M. Darghouth (ENMV, Tunisia) and are currently being used in experimental cross-immunisation trials prior to selection of a suitable vaccine candidate to be used in the field in Tunisia.

#### 1.4 DIVERSITY IN PROTOZOAN PARASITES

Various methods have been employed to characterise protozoan parasites, ranging from morphological studies to DNA investigations. Given the general similarities between many of the protozoan parasites, the results of applying such methods to a particular parasite have general implications for the genetics and biology of protozoan parasites in general. The study of protozoan genetics is complex and investigations of both the genome and the parasite life cycle are needed, using both molecular and biological data, to obtain a better understanding of the process involved in generating diversity. In this section, literature on diversity in *Theileria* and four other protozoan parasites is reviewed. These investigations into parasite diversity were made at both the phenotypic and genomic level and thus provide models for the present study.

Firstly, studies on trypanosomes illustrate the use of isoenzyme analysis, DNA probes, protein variation and chromosome size polymorphism. These studies were aimed at developing new taxonomic tools studying the origin of the variation observed and examining the parasite biology in relation to ploidy. Secondly the diversity described in *Leishmania* parasites illustrates the importance of isoenzymes in the characterisation of the different taxa,

each defined by a specific zymodeme. DNA techniques have also been used to further clarify the classification within the genus and to investigate the ploidy of the parasite and the mechanisms generating diversity. Thirdly, the importance of studies on diversity in the design of a live vaccine are illustrated for *Babesia* parasites. These include studies on virulence/attenuation and tick-transmissibility of different strains, together with studies on the mechanisms involved. Fourthly, the example of *Plasmodium* is examined; the studies described mainly emphasise the extent of genetic diversity in this genus and its importance in relation to the design of a successful vaccine against one of the most important human disease. Lastly the limited data on diversity in *Theileria* parasites are described and the rationale behind the present study is presented.

#### **1.4.1 *Trypanosoma***

A detailed study on enzyme polymorphism in trypanosomes and its relevance to both epidemiology and taxonomy has been made by Gibson *et al* (1980). They studied 160 *Trypanozoon* stocks of diverse origins using 12 isoenzymes. Each stock was assigned to a particular zymodeme according to its combination of electrophoretic patterns. As a consequence the stocks were divided in 59 zymodemes. Little or no variation between stocks was found for six enzymes, while the results based on analyses of the other six enzymes showed that, while there were differences between West and East African *T.brucei* stocks in the frequency of particular alleles, no clear cut distinction could be made. The authors also suggested that *T.evansi* should be included in the *T.brucei* group, since it was closely related to other parasites of this group. They also confirmed that the three *T.brucei* subspecies (namely *T.b.gambiense*, *T.b.rhodesiense* and the non-human infective *T.b.brucei*), are closely related and suggested that they do not warrant sub-specific status. The only way to distinguish *T.b.brucei*, from the two other subspecies in the laboratory, is by its sensitivity to lysis by human sera (Cross, 1990).

A similar investigation, using DNA probes, was carried out by Hide *et al* (1991), to study the relationships between stocks of *T.b.rhodesiense* from Kenya, Uganda and Zambia. The authors showed that Zambian isolates are different from Kenyan/Ugandan isolates. They

also showed a particular close relatedness between human serum resistant stocks isolated from the Kenya/Uganda focus and inferred that these might have arisen by clonal selection from a common source.

Another important aspect in the biology of trypanosomes is their ability to undergo antigenic variation, which appears to be the primary mechanism for evasion of the host's immune response (Gray and Luckins, 1976). This variation is effected through the sequential expression of variant surface glycoproteins (VSGs). These act as a replaceable surface coat which specifies the variable antigen type (VATs) of the trypanosome. The new VSGs are responsible for the waves of parasitaemia in the blood. More than 100 VATs have been derived experimentally from a clonal infection (Barry and Turner, 1991).

Three molecular mechanisms are thought to be responsible for this antigenic variation in trypanosomes:

- (i) Activation of a minority of already existing telomeric VSG genes.
- (ii) Activation of genes localised within the chromosomes by transposition of a copy into one of a number of expression sites.
- (iii) Recombinational events between the expression site and other loci containing an inactive VSG gene. This last, however, is thought to occur at a very low rate.

The different routes overlap in timing but a basic VSG expression hierarchy involves telomeric genes first followed by internal genes (Barry and Turner 1991). VSG genes are thought to have evolved by duplication and mutation of ancestral genes, coupled to the development of a system for regulating their expression; VSG genes encoding about 1000 distinct VSG-related sequences, are thought to exist per genome (Cross, 1990).

Hence variability in trypanosomes involves both diversity between parasite stocks and antigenic variation within individual trypanosomes. Antigenic variation is thought to be a means of prolonging infection, so the parasite has time to be transmitted to the invertebrate vector.

The study of stock variability in trypanosomes is of certain interest in assessing the



degree of similarity of stocks from different foci, or from the same focus at different periods of time.

Part of the diversity detected in trypanosome stocks could be explained by the genetics of these parasites. It has been demonstrated, using laboratory crosses, that genetic exchange and mating occurs in trypanosomes (Tait, 1980; Jenni *et al*, 1986). The occurrence of genetic exchange helps to explain the considerable polymorphism in the electrophoretic patterns observed in isoenzyme studies. Tait and Turner (1990) reviewed the present state of knowledge regarding genetic exchange in *T.brucei*. The electrophoretic patterns for each enzyme were shown to be the result of allelic variation at each locus. Similar variation was also found using RFLPs detected by single copy gene probes. Using laboratory crosses of two parent stocks, homozygous and different from each other at several loci, hybrid individuals containing genetic material from each parent were produced which remained heterozygous after cloning (progeny clones). However it was shown that genetic exchange is not obligatory during mating and only a proportion of flies produced hybrid trypanosomes, the remaining clones being identical to one or other of the parents. The analysis of markers, heterozygous in one or both parents, in progeny clones derived from laboratory crosses, indicated that genetic exchange in trypanosomes involves meiosis, allelic segregation and recombination (Tait and Turner, 1990).

*T.brucei* parasites have been described as diploid by indirect evidence from isoenzyme data, RFLP's of single copy genes and DNA content (Tait and Turner, 1990). However the measurement of the copy number of specific variant antigen genes, for many of them present as a single copy per genome, suggests that they are in fact haploid (Borst and Cross, 1982). An interpretation of these observations was made by Tait and Turner (1990). These authors suggested that the chromosomes in *T.brucei*, in particular, can be divided into three classes. The large chromosomes (700Kb-5.7Mb) as determined by their mobility in PFGE, are probably diploid while the mini (50-150Kb) and the intermediate (200-700Kb) chromosomes are haploid. Homologous large chromosomes are not necessarily of equivalent size and therefore generate polymorphism between the different parasites at least in the karyotype.

The precise mechanism of the genetic exchange and sexual process in trypanosomes remains undetermined. The principal limitation to this is the difficulty in generating sufficient numbers of progeny clones for analysis.

#### 1.4.2 *Leishmania*

The main objectives of investigations into diversity in *Leishmania* are the identification and characterisation of the parasite for epidemiological and clinical purposes. At the same time questions regarding the parasite life cycle and biology such as ploidy have been addressed. The main method used in the identification of *Leishmania* spp, is isoenzyme analysis. This enables stocks to be distinguished on the basis of their zymodeme and permits differentiation between strains (Chance, 1979). Le Blancq *et al* (1986) showed that classifications based on isoenzyme data are still valid when additional characters are introduced and when stocks from different origins are used. This study also showed that the numerical analysis of the zymodemes of "Old World" *Leishmania* reveals substantial inter-group divergence and low intra-group variation in accordance with the integrity of the different taxa. However separate species status is given to some *Leishmania*; e.g. *L.gerberilli*, two *Leishmania* spp isolated from Namibia and an isolate from the former U.S.S.R. (Le Blancq *et al*, 1986). The rest of the groups of zymodemes exist in accordance with taxa already established: *L.major*, *L.tropica*, *L.aethiopica* and *L.donovani* (*L.donovani sensu stricto* and *L.infantum*). These subdivisions are usually considered as separate species.

DNA-based techniques have been investigated for their potential as tools for identifying *Leishmania* spp (Barker and Butcher, 1983). Kinetoplast DNA (kDNA) has been studied and differences in restriction fragment patterns and hybridisation have been demonstrated between the various taxa (Spithill and Grumont, 1984). DNA restriction and Southern blotting analyses have also shown that RFLP patterns of isolates belonging to the same taxa exhibit differences. Studies on the kDNA thus seem to have revealed differences between strains rather than similarities (Spithill and Grumont, 1984). Studies on *Leishmania* were aimed at examining the relationship between different stocks for a variety of clinical and epidemiological investigations, and so additionally research was initiated on nuclear DNA

(Barker *et al*, 1986; Van Eys *et al*, 1989). These studies were directed towards sequences of nuclear DNA that have a high degree of homology within a taxon but show restriction polymorphism between taxa. These sequences when used in Southern blotting, will detect differences in restriction sites between taxa and will provide variations in banding patterns, whereas homology within a taxon will emphasise close relationships between strains.

Sequences specific for each taxon or small number of strains could be used to detect and identify *Leishmania* in assays such as dot blots or polymerase chain reaction (PCR) followed by identification of the products by agarose gel electrophoresis (Van Eys *et al*, 1989).

The pathways resulting from infection by different members of the *Leishmania* genus varies widely. Infections by parasites from the *L.donovani* complex are systemic whereas other infections from the Old World taxa are generally localised (Le Blancq and Peters, 1986). The characterisation of *Leishmania* species improves the diagnosis and provides a better basis for clinical decisions concerning initiation of chemotherapy (Spithill and Grumont, 1986). Ideally the methods of identification should provide a classification that has a direct relation to the pathology. Two parameters are used to achieve this, isoenzyme analysis and DNA probes.

Studies using genomic DNA probes have shown that sequences that hybridise to the Old World *Leishmania* and *L.chagasi* do not in general hybridise with the "New World" *Leishmania* and vice versa (Van Eys *et al* 1989, 1991). In the same studies the authors showed that strains from the same taxon but from different geographical regions show the same pattern of hybridisation with specific probes. Thus, genomic DNA does not seem to be as diverse as kDNA in *Leishmania* for stocks from the same taxon but isolated from different regions. Such studies have also shown that some isolates, which did not fit in one of the existing zymodemes, could, using DNA probes, be classified into one of the taxa.

Van Eys *et al* (1991) selected a genomic DNA probe from a library of *L.major* which recognised the old world *Leishmania* and distinguished between *L.infantum*/*L.chagasi* and *L.donovani* from amongst some 50 strains within these species. The results of this study supported the hypothesis that *L.chagasi* is in fact *L.infantum*.

The genome of *Leishmania* has also been studied at the chromosome level. Pulse field gel electrophoresis (PFGE) has shown extensive size polymorphism of homologous chromosomes amongst different strains and between different species (Bastein *et al*, 1992). The polymorphism was detected in the size, the staining intensity and the number of the chromosomal bands, to the point that the karyotype is almost strain specific (Pages *et al*, 1989). Specific chromosomes in *Leishmania* need to be identified by specific DNA probes as the extreme size polymorphism between homologous chromosomes from different strains makes direct identification difficult. Consequently the exact number of chromosomes in *Leishmania* is not known (Blaineau *et al*, 1991). Sixteen different chromosomes have been identified by specific DNA probes out of 25 bands seen in *L.infantum* and 17 out of 24 in *L.major* (Bastein *et al*, 1992).

Whether *Leishmania* is diploid or haploid is still controversial, some of the isoenzyme polymorphism observed are consistent with those expected in a diploid organism capable of genetic exchange (Le Blancq *et al*, 1986). Pages *et al*(1989) detected a high degree of polymorphism in the molecular karyotypes of 21 strains and 14 clones of *L.infantum* from a restricted endemic area, using PFGE. The variation related to both the size of chromosomes and their number. This polymorphism did not correlate with isoenzyme analysis. It was suggested that the chromosome size polymorphism involves DNA amplification/deletion events rather than interchromosomal rearrangements such as translocation. Blaineau *et al* (1991) showed that size polymorphism in small chromosomes of *L.infantum* is partly due to variations located in the terminal regions suggesting instability of the subtelomeric regions. The study also described chromosome size variation in the central region of these chromosomes, probably resulting from a gene amplification by duplication. Another possibility would be the insertion of foreign DNA such as mobile genetic elements (Blaineau *et al*, 1991).

Pages *et al* (1989) also showed that *L.infantum* populations have a polyclonal structure, with one clone predominant in PFGE analysis and a close relationship between the clones. The variation noticed in the chromosome numbers between biologically closely related strains could be explained by two hypotheses; either that the parasite is diploid, or

that chromosome duplication has occurred, followed by size modification, leading to aneuploidy with homologous chromosomes having different sizes. The current general view is to consider *Leishmania* as a diploid organism (Blaineau *et al* 1991, Bastein *et al*, 1992). Support of diploidy came from a naturally found hybrid strain of *L.major* and *L.arabica* that is heterozygous for four isoenzymes and six DNA probes (Kelly *et al*, 1991). However diploidy in *Leishmania* generally means having two sets of almost identical chromosomes. Genetic exchange may occur in *Leishmania*, involving conjugation between unrelated clones and giving rise to heterozygous individuals (Bastein *et al*, 1992).

#### 1.4.3 *Babesia*

The close relationship between *Babesia* and *Theileria* (*Piroplasmorida*, *Apicomplexa*) and their morphological similarity explain the early denomination of bovine piroplasmosis used for theileriosis as well as babesiosis (Sergeant *et al*, 1945).

*Babesia bovis*, the causative agent of bovine babesiosis, is the most studied of several species of the tick-transmitted *Babesia*. Babesiosis is a disease of great economic importance in parts of the world where the tick vector, *Boophilus microplus* and other *Boophilus spp* occurs (McCosker, 1981). The concern in bovine babesiosis was the variation in virulence exhibited by different field isolates. Strains of *B.bovis* isolated from field outbreaks produce a debilitating disease in cattle which is often fatal. These parasites are also highly virulent for the tick vector (Kahl *et al*, 1983). Studies were carried out to investigate the polymorphism of *Babesia* parasites, mainly *B.bovis*, in order to understand the mechanisms involved in virulence and in the variation of tick-transmissibility. Attenuated live vaccines are currently used against babesiosis. Parasite diversity was therefore also studied in relation to parasite attenuation and mechanisms of immunity in order to assist in the design of successful vaccines.

It has been reported that there is extensive heterogeneity between and within natural isolates of *B.bovis* (Dalrymple, 1990). Virulence has been shown to be reduced by irradiation of infected blood, long-term residence in carrier cattle or multiple syringe passage through splenectomised calves (Kahl *et al*, 1983). Avirulent parasites, attenuated through calves, are



presently used for vaccination in cattle in Australia (Callow, 1979). Recent attempts to produce a non-living *B.bovis* vaccine have not been successful because they did not provide satisfactory immunity by comparison to that obtained by attenuated live vaccines (Timms *et al*, 1990).

The vaccine strains currently used in Australia are produced by serial blood passage in splenectomised cattle. However reversibility to full virulence of such strains of *B.bovis* may be obtained by rapid syringe passage of the parasite through intact calves (Callow *et al*, 1979). Vaccine strains are also infective for ticks and might revert to virulence after their passage through the invertebrate vector (Cowman *et al*, 1984b). Syringe passages in splenectomised calves also influences the virulence and infectivity of *B.bovis* for *Boophilus microplus* (Kahl *et al*, 1983).

Little is known of the molecular events associated with changes in virulence and tick transmissibility of *B.bovis*. Several authors have studied the virulent and avirulent (vaccine) forms of *B.bovis* designated K<sub>V</sub> and K<sub>a</sub>. They were characterised by differences in their proteins and antigens (Kahl *et al*, 1982), while at the DNA level, Cowman *et al* (1984a), identified a polymorphic locus, designated Bab R, that can be used to distinguish different *B.bovis* isolates and strains. Kahl *et al* (1983) confirmed the reversion to virulence of the K<sub>a</sub> strain after passage in intact calves and ticks. But these authors were not able to determine whether differential gene expression within a population and/or selection of subpopulations from the mixed parent strain, influences the expression of variable *B.bovis* protein antigens characterising the K<sub>a</sub> and K<sub>V</sub> strains.

Southern blotting of restriction digests of genomic DNA using cDNA clones, showed that most isolates of *B.bovis* contain a mixture of subpopulations and that selection of these occurs during the process of attenuation (Cowman *et al*, 1984b). While the functions of the isolated genes are not known, polymorphisms detected by these probes allowed each independent geographical *B.bovis* isolate to be distinguished. Analysis of cloned genomic fragments, homologous to one of the cDNA clones, has shown that rearrangements have occurred within this region of the genome (Cowman *et al*, 1984a). The results presented in these studies by Cowman *et al* (1984a, 1984b) suggest that a selection of subpopulations



present in some isolates can occur during attenuation or passage through ticks but it remains entirely possible that changes in gene expression are also occurring at the same time.

In order to provide a better understanding of the virulence and immunogenicity mechanisms in this parasite, several groups have produced cloned lines of *B.bovis*. Gill *et al* (1987) used an *in vivo* limiting dilution technique to produce *B.bovis* cloned lines. None of the cloned lines appeared to give quite as strong immunity as the parental K<sub>a</sub> isolate, suggesting that a combination of different parasite populations is required to provide adequate immune protection against *B.bovis*. The K<sub>a</sub> isolate was also shown to contain both virulent and avirulent subpopulations, which suggests that the difference between the K<sub>a</sub> and K<sub>v</sub> isolates is in the relative proportion of subpopulations that vary in virulence. However, in the same study, it was also observed that an apparently clonal *B.bovis* population gave rise to both avirulent and virulent lines, suggesting that virulence may be a modifiable characteristic of the parasite. Gill *et al* (1987) suggested that transmissible subpopulations may be capable of supporting transmission of subpopulations that are not transmissible by ticks, by providing some factors required for transmission.

The study of Timms *et al* (1990), using *B.bovis* cloned lines, confirmed that the K<sub>a</sub> isolate is composed of both virulent and avirulent subpopulations. Full reversion to virulence of the parasite in intact calves was as pronounced in avirulent clones as it was in the K<sub>a</sub> strain. However, while the K<sub>a</sub> strain reverted fully to virulence after its passage through *B.microplus* ticks, using splenectomised calves (a reversion to virulence similar to that obtained by blood passage in intact cattle), none of the *B.bovis* clones, or a mixture of these, were tick transmissible. The authors suggested that these clones were not of separate sexual type and that both types are required for sexual recombination and for successful vector transmission (Timms *et al*, 1990).

Results from these studies suggest that the virulence in *B.bovis* is readily modifiable and may involve alterations in the expression of specific genes, since avirulent clones may revert to virulence. It was inferred that the virulent gene(s) must still be present, but not expressed, in both the K<sub>a</sub> strain and avirulent cloned lines (Timms *et al*, 1990).

The inability of *B.bovis* cloned lines to be passaged biologically through the tick



vector *B.microplus* appears to be a stable feature. It is argued that it could provide cloned lines with certain advantages over the K<sub>a</sub> strain as vaccine because they can not be transmitted by ticks naturally in the field (Timms *et al*, 1990). The impact of such a situation on the enzootic stability of a disease, is discussed with regards to theileriosis in Section 1.4.5.

Significant problems were recently observed with the K<sub>a</sub> vaccine, with increasing numbers of challenged vaccinated animals exhibiting severe pathology (Dalrymple, 1992). It was suggested that the use of one line may have exerted selective pressure on the natural population to select for parasites able to break through the immunity induced by the K<sub>a</sub> isolate. Again this emphasises the importance of strain diversity and strain variation in the design of a successful vaccine against *B.bovis*. Thus, not only the nature of the expressed antigens, but also the environment in which they are expressed, may play a role in effective immunisation by the live attenuated vaccines (Dalrymple, 1992). Therefore it is of considerable importance to use both phenotypic and genotypic analysis of populations and well characterised cloned lines of parasites to determine the importance of variation in the vaccine and challenge strains of *B.bovis*.

#### 1.4.4. *Plasmodium*

Because of the importance of malaria, caused by *Plasmodium falciparum* in man, the study of diversity in this parasite, and to a lesser extent other species of *Plasmodium*, has been the most extensive within the parasitic protozoa.

The development of a sub-unit vaccine capable of protecting people living in endemic areas is the main priority behind any studies on malaria parasites. Immunity in malaria is poor and strain specific. Diversity was therefore studied in attempts to relate parasite variation to immunogenicity of different parasites in the field, using immunogens which occurs naturally. The emergence of drug-resistant parasites has also stimulated research towards the identification of specific genes coding for such resistance. The understanding of the mechanisms generating the polymorphism in malaria parasites was greatly facilitated by genetic crossing experiments. The studies on malaria were also aimed at elucidating the role of diversity in the evasion of the host immune system by the parasite.

*P.falciparum*, in endemic areas, causes repeated episodes of fever and parasitemia in susceptible hosts. The clinical symptoms observed vary from mild disease to severe manifestations of disease such as cerebral malaria. The disease evokes a poor immunity since residents in endemic areas often suffer repeated infections and it is only after several years of exposure to the parasite that they acquire a partially protective immunity (Mendis *et al*, 1991). The immunity is non-sterile, leading to a state of premunition (similarly to tropical theileriosis) with a very low parasite densities and the immunity acquired in one area may not be effective against parasites present in another (as observed in infection caused by *T.parva*) (Coppel *et al*, 1985). These observations implied that the immunity is strain specific and that immunological differences between parasites are prevalent in nature (A.Hill, personal communication). Subsequent studies were therefore directed towards the investigation of the extent of diversity in the *P.falciparum* population and the role of variation in the population genetics of the parasite during malaria infection.

The typing of *P.falciparum* was first conducted at a phenotypic level, using isoenzyme electrophoresis (Carter and McGregor, 1973), two-dimensional electrophoresis of parasite proteins (Tait, 1981), antigenic diversity recognised by monoclonal antibodies (McBride *et al*, 1982) and susceptibility to anti-malarial drugs such as chloroquine and pyremethamine (Thaitong *et al*, 1984). It was clear from these analyses that considerable genetic diversity exists within *P.falciparum* populations, assuming that the characters studied at a phenotypic level were under genetic control.

Strain characterisation by direct analysis of the parasite genome showed differences between species of *Plasmodium* as well as between different isolates of *P.falciparum* (Coppel *et al*, 1985). Polymorphism in malaria has also been studied for a number of genes by the application of recombinant DNA technology. Most of the genes studied encode polypeptides that are antigens during natural infection. This particular emphasis is due primarily to the interest in the development of a recombinant subunit vaccine.

Genetic diversity in *P.falciparum* has been extensively reviewed by Kemp *et al* (1990). From the studies reported, it is clear that diversity in the genes cloned will be of major importance in the design of effective vaccines. These studies also showed that most of

the antigens investigated contain tandem repeats of oligopeptide sequences varying from one isolate to another, and these sequences appear to act as dominant natural immunogens.

In addition to this form of diversity, recent studies using PFGE, have shown a considerable variation in chromosome size of parasite clones. The mechanisms involved in this phenomenon are largely due to recombinational expansion and contraction of sub-telomeric sequences. Since many of the antigens containing repetitive epitopes are encoded by sub-telomeric genes, it was suggested that there might be a possible connection between antigenic diversity and chromosome variation (Kemp *et al*, 1990).

Another form of diversity was shown to be due to the generation of novel genotypes during transmission of mixed infections (Walliker, 1989). Crossing experiments were conducted by feeding mosquitoes on a mixture of genetically different cloned parasite lines. These experiments are at the basis of genetic studies and provide data on recombination between loci and segregation of alleles. Such genetic studies can be used to analyse the genetic basis of phenotypic variation and determine whether the variation observed is due to a single gene or whether it is due to several genes at different loci in the genome.

Genetic crosses, in malaria parasites, have shown that recombination is a frequent event occurring during meiosis, shortly after the zygote formation in mosquitoes. These experiments have also indicated that progeny from cross-fertilisation events were favoured over progeny from self-fertilisation (Walker-Jonah *et al*, 1992). The distinction between oocysts which are the products of self-fertilisation events, from those which are the products of cross-fertilisation between different parasite clones, was shown to be possible by the use of PCR technique on DNA obtained from these oocysts (Ranford-Cartwright *et al*, 1991).

Previous studies on malaria parasites had provided evidence for haploidy of the parasite. DNA studies provided additional evidence showing that the only diploid stage is the zygote, produced by fertilisation of gametes in the mosquitoes (Kemp *et al*, 1990).

Genetic studies in malaria were used to investigate variation in antigen genes. It is uncertain whether the polymorphism found in putatively protective antigens in malaria contributes to evasion of the host immune response. The fact that the highest degree of antigenic polymorphism has been found in stages of the parasite exposed to the host immune

system, in natural infection, is in favour of such a correlation (Mendis *et al*, 1991). Variation does not extend to all parasite antigens in malaria and parasite molecules that are critical for biological functions are probably conserved (Mendis *et al*, 1991). Variation is also observed in parasite antigens located on the surface of infected erythrocytes. These antigens may represent good targets for the immune system (Mendis *et al*, 1991). It was shown that these antigens are not only variant in different isolates but also undergo variation during the course of natural infection with a cloned parasite. The structures of these antigens and the importance of the molecular mechanisms involved are still unknown.

Snewin *et al* (1991) analysed the polymorphism of the alleles of the merozoite surface antigens, MSA 1 and MSA 2 in *P.falciparum* wild isolates from Colombia, where the endemicity is low. The analysis showed that one allele of MSA 1 was found in the majority of the isolates and that a number of isolates were identical for MSA 2 alleles despite being collected from dispersed areas. The authors suggested that more variation could be found in wild isolates collected from highly endemic regions if the diversity has evolved under the selection pressure of the host immune response.

The remarkable plasticity of the genome in malaria parasites is based primarily on several mechanisms involved in the generation of chromosome size polymorphism. It was inferred that the polymorphism found could be associated with programmed DNA rearrangements involved in the generation of gene transcription or in generating diversity in genes coding for proteins recognised by the immune system of the host, enabling the parasite to evade it (Vernick *et al*, 1988). This, however, is not accepted by all authors; Janse (1993) argued that novel sized chromosomes, occurring during mitotic multiplication, are generated by aberrant DNA rearrangements that also occur in the DNA of other eukaryotes. This author also suggested that this phenomenon often affected parasite viability under natural conditions and that similar rearrangements have been described in cancer cells or cells exposed to environmental pressure *in vitro*.

#### 1.4.5 *Theileria*

Investigations into diversity in *Theileria* have followed conventional methods used

for other protozoan parasites, some of which have been described. They have included studies on biochemical polymorphism expressed by isoenzymes, variation of protein antigens and repetitive and non-repetitive sequences in the genome (using DNA probes), and, recently, chromosome size polymorphism. Examples of these will be illustrated and discussed below.

Early studies on *T.annulata* demonstrated, through variation in virulence and cross immunity, that differences existed between *Theileria* strains (Barnett, 1963). From studies to define the levels of virulence in cattle of different field strains of *T.annulata*, it was suggested that the ability of *Theileria* strains to induce immunity against one another is not associated with their virulence but their antigenic diversity (Pipano *et al*, 1974). While the rule is that cross-immunity exists between parasites from different parts of the Indian sub-continent (Gill, *et al* 1980), a further study conducted in India (Subramanian, 1991), inferred that failure in vaccination might be due to the existence of immunological variants.

These studies, and similar ones on other protozoan parasites, have stimulated research into diversity in *T.annulata*, despite the existence of satisfactory vaccines in some countries (Pipano, 1990). In the case of *T.parva*, where cross-immunity between different stocks is highly variable a study of parasite diversity with the main aim of being able to predict cross-immunising ability, was considered a priority. With the development of new technologies, diversity in *T.parva*, has been investigated, with the aim of providing a better understanding of parasite biology. This, in turn, was hoped would lead to better selection of potential vaccine stocks (Norval *et al*, 1992). Phenotypic analysis was undertaken using MAbs raised against different stages of the parasite. Monoclonal antibodies reacting with schizont-infected cells have detected antigenic diversity in *T.parva* (Pinder & Hewett, 1980) and *T.annulata* (Shiels *et al*, 1986a) and offered the first method for strain characterisation. When different stocks of parasite are tested against a panel of MAbs, a profile of reactivity can be defined for each stock. These antigenic profiles appear to be fixed characters when the parasites are passaged through ticks (Irvin, 1987). MAbs were also the first markers used to detect the presence of mixed parasite populations (Shiels *et al*, 1986a). Such schizont-infected cell lines would show a variable percentage of reactivity with some of the MAbs.



Cloned cell lines deriving from these cell lines segregated into reactive positive clones and non-reactive negative clones. These clones retain their profile of reactivity upon passage. Some of these MAbs are stage specific whereas others recognise common determinants in some or all parasites stages. However it is still not possible to predict the cross-immunising capability of a *T.parva* isolate from its MAb profile. The MAbs raised so far against *Theileria* parasites are valuable markers of strain differences but do not distinguish specifically between parasites of the *T.parva* subspecies: *T.p.parva*, *T.p.bovis* and *T.p.lawrencei* (Conrad *et al*, 1989). As a consequence of these results, and also of other phenotypic and genomic studies, this classification has been abandoned (Norval *et al*, 1992).

Phenotypic characterisation of theilerial proteins by two-dimensional gel electrophoresis was also reported by several groups. This method showed only minor differences between parasites of the *T.parva* complex and the three subspecies could not be reliably distinguished according to their protein differences (Sugimoto *et al*, 1989).

A study on *T.parva* (Shapiro *et al*, 1987), using Western blot analysis on schizont extracts, revealed the presence of a polymorphic immunodominant protein amongst different parasite stocks. Antigenic diversity was also used to distinguish between a group of less virulent species of *Theileria* occurring mainly in Japan, Australia and Britain, namely *T.sergenti*, *T.buffeli* and *T.orientalis*. These were reported as belonging to one species and the name *T.orientalis* was proposed (Uilenberg *et al*, 1985). However studies by Japanese workers have indicated that the parasite group should be divided into *T.sergenti* (Japan) and a *T.buffeli/orientalis* complex. The dichotomy is based on the results of transmission experiments using various species of tick vector (xenodiagnosis), protein analysis of piroplasms by two dimensional gel electrophoresis and, recently, the results of serological comparison using a comparative ELISA and Western blotting in combination with two-dimensional gel electrophoresis (Kawazu *et al*, 1992). The comparative ELISA using sera from cattle that had been infected singly with each of the putative subspecies of parasites, revealed close relationships between the three parasite types. The separation of *T.sergenti* from the *T.buffeli/orientalis* was mainly based on the detection by immune sera of different proteins on the two-dimensional gels. These immunodominant proteins in piroplasms from

infected cattle were of 33kDa in *T.sergenti* and 34kDa in *T.buffeli* and *T.orientalis*, although serological cross-reactivity between these molecules was detected. It was suggested that these 33/34kDa proteins might represent homologous molecules with a partially different peptide sequence or differential glycosylation (Kawazu *et al*, 1992). Proteins of a similar molecular weight and strongly detected by immune sera have also been detected in piroplasms of *T.annulata* and *T.mutans*. In *T.annulata* a study by Dickson and Shiels (1993) showed the presence of two forms of the protein in genotypically different cloned cell lines, with molecular masses of 30kDa and 32kDa. In *T.mutans* the 32kDa protein has been used as the basis of an antigen detection ELISA (Katende *et al*, 1990).

Diversity in *Theileria* isolates has also been studied using parasite enzyme polymorphism. Differences between isoenzyme patterns of different strains are associated with differences in electric charge. The different forms of specific enzymes are probably controlled by nuclear genes and remain stable over many passages (Irvin, 1987). In *Theileria* species the most variable enzymes were found to be glucose phosphate isomerase (GPI) and hexokinase. GPI was used to differentiate strains of *T.annulata* (Melrose *et al*, 1984) but with *T.parva* no clear differences between different stocks were detected (Allsopp *et al*, 1985).

Polymorphism has also been detected at the genetic level in *Theileria* species. A number of gene clones have been identified, coding for parasite antigens, which produce proteins recognised by MAbs (Williamson *et al*, 1989). Probes corresponding to these genes, and also gene probes randomly selected from a *Theileria* genomic library (Conrad *et al*, 1987a; Allsopp and Allsopp, 1988), were also used for parasite strain characterisation. Again these methods did not discriminate specifically between *T.parva* subspecies and could not be reliably used to predict cross-immunity between stocks (Conrad *et al*, 1989). *Theileria*-specific DNA probes have not only been used to differentiate species, stocks and clones of *Theileria* but also to detect theilerial parasites in their vectors with the aim of differentiating between ticks infected with pathogenic and non-pathogenic species (Chen *et al*, 1991).

Specific gene probes have also been used to design primers for the PCR to amplify specific DNA sequences. These primers were used to improve the characterisation of



*T.parva* isolates (Allsopp *et al*, 1989) and to detect the carrier state in *T.parva*-infected cattle (Bishop *et al*, 1992). The PCR technique has the advantage of detecting the parasite specifically without the need for isolation *in vitro* and is capable of detecting parasites at very low parasitaemias, such as in the carrier state, or early in the disease, when serological responses to schizonts are not yet detectable.

Chromosome size polymorphism in *Theileria* has recently been investigated using PFGE and hybridisation with a *Plasmodium* telomeric repeat probe (Morzaria *et al*, 1990). The results provided useful information on the genomic diversity at intra and inter-specific level. They showed polymorphism between and heterogeneity within various stocks of *T.parva* and suggested that most of the polymorphism and heterogeneity analysed at the chromosome level occurred in the telomeric and subtelomeric regions of the genome. Similar analyses also showed that the *T.parva* genome possesses four chromosomes (Morzaria *et al*, 1992a). Analysis of karyotype variation was used by Morzaria *et al* (1992b) to provide evidence for a sexual cycle in *T.parva* and genetic exchange between two cloned parasite cell lines generating recombinant parasites. As inferred for other protozoan parasites, this mechanism might be partly responsible for the polymorphism found in *Theileria* parasites (Morzaria *et al*, 1992a).

## 1.5 CONCLUSION

*T.annulata*, as reviewed in this chapter, presents similarities in its biology, life-cycle, the disease it causes (Section 1.2) and its epidemiology (Section 1.3) to other protozoan parasites, studies on the diversity of some of which are described in this Chapter (Section 1.4).

From the earlier work reviewed, diversity appeared to be an important component to consider in both epidemiological and immunological studies. Therefore this study describes an investigation into diversity in the pathogenic protozoan parasite *T.annulata*, since this has not been attempted before in any comprehensive manner.

Studies on other protozoan parasites and *Theileria* have shown that MAbs which detect protein variation, polymorphic isoenzymes and DNA probes which detect genomic

diversity, were the tools most frequently and successfully used to assess the extent of diversity in parasite populations. Accordingly, these were the methods chosen for this study whose primary objective was to investigate population diversity in *T.annulata* in Tunisia. This analysis was conducted with the following objectives:

- (i) To focus on one geographical region, in this case one country.
- (ii) To assess the degree of polymorphism in defined regions and to examine whether the diversity is localised to some of these regions or whether high levels of variation exists in a restricted area such as a small village as has been found with *P.falciparum* (Babiker *et al*, 1991b).
- (iii) To generate tools to analyse this polymorphism and use these to investigate the basis of variation i.e. genetic exchange and recombination.
- (iv) Finally to use the information obtained to assist in selecting the most appropriate stock to be used as a vaccine in Tunisia.

## *CHAPTER TWO*

### *GENERATION OF PARASITE MATERIAL*

## **2.1 INTRODUCTION**

This chapter describes the general materials and methods used to generate parasite material. The techniques employed in the isolation, maintenance, cloning, cryopreservation and resuscitation of the parasite are detailed. The methods used to obtain the different life-cycle stages of the parasite are given and include (i) *in vivo* infection of calves and subsequent transmission to ticks (ii) *in vitro* establishment of infected cell lines from these infected ticks. Finally, the nature of material collected is described and discussed.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Cattle**

#### **2.2.1.1 Experimental cattle**

Friesian x Holstein male calves, 3-4 months of age were selected from farms with no theileriosis. They were screened to check that no piroplasms were present in blood smears and that they were seronegative for antibodies to *T.annulata* schizonts in IFAT (Chapter 3, Section 3.2.2.1). Only piroplasm and antibody negative calves were brought and were kept for at least one week before starting experiments. Preinfection packed cell volume (PCV) and body temperature were recorded and they were bled for preinfection serum. These animals were kept either at the ENMV or the Institut Pasteur de Tunis (IPT) farm, Tunisia.

#### **2.2.1.2 Field cattle**

Animals from different sites in the country, mainly cross breeds of Holstein or Friesian, suspected of experiencing clinical theileriosis were used to collect parasite material. They were of both sexes and wide range of ages, not always recorded by the owner or the field worker (see Section 2.3.1.2).

### **2.2.2 Ticks**

#### **2.2.2.1 Laboratory ticks**

*Hyalomma anatolicum excavatum* ticks were maintained as a laboratory colony at the ENMV. Nymphs were kindly provided by Dr M.Darghouth (ENMV) and fed in ear bags on experimentally infected calves in Tunisia (Walker *et al*, 1985)

#### **2.2.2.2 Field ticks**

*Hyalomma detritum* nymphs were collected from cracks in walls of cow sheds on farms situated in the highly endemic area of Bouhnach, nearby the ENMV (Sidi Thabet) and comprising farms of type B described in Chapter one (Section 1.3.3).

### **2.2.3 Experimental in vivo infection**

#### **2.2.3.1 Infection**

Schizont-infected cell lines used to infect calves were resuscitated and subcultured two to three times before being inoculated into calves. On the day of infection, cells from 2 day old, exponentially growing cultures in complete medium with 10% foetal calf serum (FCS) (Section 2.2.5.1) were counted and their viability assessed. The calves were inoculated subcutaneously with  $2 \times 10^6$  viable cells in the right prescapular region.

#### **2.2.3.2 Monitoring**

Rectal temperatures were recorded daily. The haematological parameters surveyed comprised, haematocrit (PCV), total white blood cell count (WBC) and the platelets count (PL). These were performed three times weekly on whole venous blood in EDTA. The PCV was measured using a haematocrit microcentrifuge and WBC and PL were measured using a Coulter counter (MAXM). The two latter parameters were only carried out for animals infected at the IPT farm.

The animals were also monitored for the presence of the parasite three times weekly. The parasitemia was assessed as the percentage of red blood cells (RBC) infected with piroplasms counted from Giemsa stained blood smears (Shute, 1966 and Appendix 1). The presence of the schizonts (macroschizont and microschorizonts) in the draining lymph node (LN) was assessed on Giemsa stained LN needle biopsy smears.

### **2.2.3.3 Collection of parasite material**

PBM were isolated from the calves on days 12-14 post-infection, in order to establish schizont-infected cell lines (see Section 2.2.5.1). These dates were shown to be the most successful dates to establish *T.annulata* infected cell lines from calves infected with cell lines (Ben Miled, unpublished observations).

When the piroplasm parasitemias exceeded 5% large amounts of blood were collected from the animals in order to separate and purify piroplasms.

One thousand nymphs were fed on selected calves in order to pick up circulating parasites. The ticks were applied to the animals on day 19 post-infection according to previous data (Ben Miled, unpublished observations).

### **2.2.3.4 Serology**

The animals were bled for serum preinfection, day 28-30 post-infection and, if challenged, post-challenge at day 60 after primary infection.

The sera were screened for anti-schizont antibodies in the IFAT (as described in Chapter 3, Section 3.2.2.1).

### **2.2.3.5 Drug treatment**

In Tunisia, buparvaquone (Butalex-Pitman-Moore) kindly provided by Ms V.Singh (Pitman-Moore) was used for the treatment of animals undergoing severe clinical reactions.

### **2.2.3.6 Challenge**

Calves infected in Tunisia were challenged with 1.6 tick equivalent (t.e) of *TaTu* 9A tick stabilate. (Kindly provided by Dr M.Darghouth, ENMV, Tunisia).

This stabilate gave 100% mortality in all susceptible animals inoculated at the dose used.

Where calves were challenged, the challenge was given between day 28 and 35 after the immunising infection, in the left prescapular region.

#### **2.2.3.7 Duration of experimental infection in calves**

The animals were monitored for 30-40 days following the immunisation and for another 30 days, if challenged, following the challenge, according to the schedule described in Section 2.2.3.2.

### **2.2.4 Sporozoites**

#### **2.2.4.1 Dissection of salivary glands**

For obtaining sterile salivary glands for *in vitro* culture infection, adult ticks were surface sterilised by immersion for 2 min in 0.2% thiomersal (BDH) in 20% ethanol, 5 mins in 1% benzalkonium chloride (Roccal, Winthrop Laboratories), 30 seconds in 70% ethanol followed by two rinses in sterile distilled water. They were then dried on sterile filter paper for 10 min.

The ticks were embedded, dorsal sides uppermost, in a sterile petri-dish containing wax and covered with Hanks balanced salt solution (HBSS, Gibco) containing 0.5% lactalbumin hydrolysate (Jones and Cunningham, 1961). They were dissected under a stereoscopic dissecting microscope (x 20 magnification) (Prior) by removal of the dorsal integument and teasing out the pair of salivary glands (Purnell and Joyner, 1968).

When ticks were dissected to assess *T.annulata* infection rate or to provide salivary gland lysates for glucose phosphate isomerase (GPI) enzyme electrophoresis and DNA extraction, complete sterility was not necessary and they were simply covered by sterile phosphate buffered saline pH 7.4 (PBS) for dissection.

#### **2.2.4.2 Staining of salivary glands.**

Salivary glands were stained with methyl green-pyronin using the procedure described by Walker *et al* (1979) and examined for the presence of infected acini. The infection rates (percentage of ticks infected) and mean number of infected acini were determined using ten male and ten female ticks per tick batch.



#### 2.2.4.3 Preparation of Ground up tick supernatant (GUTS) and GUTS filtrate (GF).

Once dropped from the animals or after collection from the walls in the field, the nymphal ticks were held one month at 28°C to moult then kept at 16°C until used. They were then incubated at 37°C for 4 days in a humidified chamber to permit maturation of the parasite in the salivary glands.

GUTS was prepared according to Brown (1983). *Hyalomma* adult ticks were surface sterilised by one rinse in Roccalt, three in 70% ethanol and four in warm minimum essential medium (MEM, Gibco) containing 200ui/ml penicillin, 200ug/ml streptomycin and 100u/ml nystatin, pH 7, the ticks being left 10 min in the last rinse. Medium was removed and the ticks were placed in a sterile mortar. They were ground in cold MEM containing 3.5% bovine plasma albumin (BPA, Sigma) using a final volume adjusted to give a final concentration of 4 t.e per ml. The ticks were initially ground in a third of the volume, this medium was removed and the grind was repeated twice using appropriate volumes of the remaining medium. The medium recovered from the grind was pooled and centrifuged at 100 x g for 5 min to remove tick debris. The supernatant resulting is known as ground up tick supernatant (GUTS). The GUTS was filtered through two filters, AP and 8µ (Millipore), to obtain the 8µ GUTS filtrate (GF). The quality and quantity of sporozoites harvested from the infected salivary glands of the ticks was assessed by making Giemsa stained cytocentrifuge smears of the GF.

#### 2.2.4.4 Stabilate preparation

Adult *Hyalomma* ticks after moult (see previous section) were fed on rabbit ears for 3 days and GUTS at 4 t.e in MEM/BPA 3.5% was prepared (as described in previous section). The GUTS was diluted with an equal volume of MEM/BPA/glycerol 15% (Analar) and mixed thoroughly to give a final concentration of 7.5% glycerol as cryopreservative to GUTS at 2 t.e per ml. This was then equilibrated for 30 min at room temperature and distributed into cryovials. The vials were placed at -70°C for 24 hr then transferred to liquid nitrogen.

The stabilates were resuscitated by rapidly thawing at 37°C and equilibrated at room temperature for 30 min before being inoculated into calves.

## 2.2.5 *T.annulata*-infected lymphoblastoid cell lines

### 2.2.5.1 Establishment

#### a) From infected animals: isolation of PBM.

Venus blood was collected by jugular puncture into 10mls vacutainers containing lithium heparin (Becton-Dickinson). After mixing the blood with an equal volume of sterile PBS, it was layered onto 8ml of Ficoll Hypaque (Pharmacia) and centrifuged at 800 x g for 30 min at room temperature. The PBM were recovered from the interface and washed in 20ml of PBS by centrifugation for 10 min at 300 x g at room temperature. The cells were washed a second time in PBS by centrifugation for 5 min at 300 x g. The supernatant was discarded and the PBM pellet resuspended in 10ml of complete medium consisting of RPMI 1640, supplemented with 2mM L-glutamine, 100ug/ml streptomycin, 100 iu/ml penicillin and 10-20% FCS (all Gibco).

Cytocentrifuge smears were prepared from the cell suspension which was transferred to a 25cm<sup>2</sup> tissue culture flask (Nunc) and incubated at 37°C in 5% CO<sub>2</sub> humidified incubator.

The cytocentrifuge smears were dried, fixed in methanol and stained in Giemsa.

#### b) By in vitro infection of PBM.

Adult ticks incubated at 37°C for four days were used to carry out *in vitro* infections. They were shown to have less mature sporozoites than when incubated on rabbit ears for three days but it was considered that this would not be of serious consequence when infecting PBM and would avoid unnecessary stress to rabbits.

PBM collected, as described above, from naive animals, were infected either with GF (Section 2.2.4.3) prepared from a batch of ticks or infected salivary glands isolated from individual ticks (Section 2.2.4.1).

PBM resuspended in complete medium with 20% FCS, were distributed in 24 well plates (Costar) at 10<sup>6</sup> cells per well in a volume of 1ml. Three dilutions of GF (1, 0.5 and 0.25 t.e per ml) in complete medium were distributed at 1ml per well using three wells per dilution. The plates were incubated at 37°C in a 5% CO<sub>2</sub> incubator and the medium changed twice weekly by removing 1ml and replacing with 1ml fresh complete medium.

Cytocentrifuge smears were taken regularly to assess whether infection was establishing. Once patently infected the cultures were transferred from individual wells to 25cm<sup>2</sup> flasks, grown up to a high infection level and aliquots cryopreserved and maintained as described in next sections.

Infection of PBM from individual ticks was carried out using sterile salivary glands (see Section 2.2.4.1). Once removed each pair of salivary glands was crushed in 2ml of 20% complete medium in Griffith tubes in order to free the sporozoites. The suspension was then distributed at 1ml per well (two wells per tick) and PBM was added to the well as described above.

#### **2.2.5.2 Maintenance**

In the early stages after PBM were isolated from *T.annulata* infected animals and put into tissue culture flasks, the medium was changed every second day. In order to change medium, the flasks were incubated vertically for 30 min, then 8ml were removed, taking care to leave the majority of cells in the flask, and replaced with 8ml fresh complete medium.

Giemsa stained cytocentrifuge smears were prepared regularly to assess whether infection was establishing.

Once greater than 50% of cell were infected with *T.annulata* schizonts, half the flask contents were transferred to a second flask. The two flasks were then supplemented with equal volume of fresh complete medium and incubated at 37°C in 5% CO<sub>2</sub>.

Once the first subculture was made with more than 50% of the cells infected, cells underwent a Log<sub>10</sub> increase every 48-72 hr (Brown, 1983). Cultures were passaged every two to three days, being reseeded at a cell density of 1-2 x 10<sup>5</sup> cells per ml, in the same or a new 25cm<sup>2</sup> flask. In this study, cells were considered to have been passaged each time medium was changed. Subcultures into other 25cm<sup>2</sup> flasks or larger flasks were made by seeding as above.

#### **2.2.5.3 Cryopreservation and resuscitation**

Cells from two day old cultures were centrifuged at 300 x g for 5 min at room temperature. The supernatant was discarded and the pellet resuspended in cold 20% complete medium at approximately  $2 \times 10^7$  cells per ml. An equal volume of 20% dimethyl sulphoxide (DMSO, BDH) in cold 20% complete medium was added drop wise while agitating the cell suspension with the pipette end. Once the cells were mixed at a final concentration of 10% DMSO, they were dispensed into chilled 1ml cryovials (Nunc), transferred to -70°C freezer for 24 hr and then into liquid nitrogen.

Cells were resuscitated by rapid thawing in 37°C water bath, then resuspending the vial contents in 10ml PBS. This diluted cell suspension was then centrifuged at 300 x g for 5 min at room temperature, the supernatant discarded and the cells resuspended in 10ml of fresh complete medium. This final, resuscitated cell suspension was put into 25cm<sup>2</sup> flasks (10ml/flask) and incubated at 37°C, 5% CO<sub>2</sub>.

#### **2.2.5.4 Limit dilution and cloning**

Cells for cloning were harvested from cultures 48 hr after passaging and cell concentration and viability were assessed. The cells were centrifuged at 300 x g for 5 min at room temperature, resuspended in 20% complete medium and diluted to 10 cells per ml. Three ml of the cell suspension were mixed with 17ml of 20% complete medium to give an average of 0.3 cell per 200ul. The cell suspension was then distributed into 96 well flat bottomed microtitre plates (Nunc) at 200ul per well and the plates incubated at 37°C in 5% CO<sub>2</sub>.

The plates were checked for the presence of growing clones on day 6 using an inverted microscope. Once clones were observed to be growing, half the medium was changed without disturbing the growing clones. This stage was usually reached 8-12 days after cloning. When the clones filled most of the well, they were transferred to wells of a 24 well plate in 2ml of 20% complete medium and subsequently expanded into a 25cm<sup>2</sup> flask.

Cloned established cell lines were maintained, cryopreserved and resuscitated as described above but using always 20% complete medium.

### 2.2.6 *Piroplasm isolation*

One hundred ml of blood was taken aseptically by jugular puncture into 1ml (1000 UI) of lithium heparin (Liquemine-Roche).

Blood from field cases was transported to the laboratory as rapidly as possible. It was centrifuged at 2500 x g for 15 min at 4°C. The plasma and buffy coat were removed with the top 5% of erythrocytes. The RBC were then washed three times with cold PBS as above, discarding the top 5% erythrocytes each time to minimise contamination with bovine leucocytes. The packed erythrocytes were then lysed at room temperature with three volumes of prewarmed (37°C) 0.9% ammonium chloride in H<sub>2</sub>O. After 3-10 min, when the colour of the blood changed to deep red, the lysed erythrocytes were centrifuged at 800 x g for 5 mins at 4°C in order to remove most of the remaining leucocytes, their cell nuclei and the unlysed erythrocytes.

Cytocentrifuge smears were made from the pellet and the supernatant. The supernatant containing the piroplasms freed from lysed RBC was transferred to 30ml sterile polypropylene tubes (Sigma) and centrifuged at 7000 x g for 20 mins at 4°C. The supernatant was discarded and the pellet containing the piroplasms washed three times with cold PBS as above.

The final piroplasm pellet was aliquoted into 1ml cryovials and stored at -70°C. A cytocentrifuge smear was made from the final pellet and stained in Giemsa to assess the quality of the parasite material collected.

In ideal conditions only nuclei with leucocytes and unlysed RBC were seen in the pellet obtained from the first slow centrifugation after lysis. However, if the material was found to be rich in packed piroplasms, it was cryopreserved at -70°C.

### 2.2.7 *Terminology employed for parasite material.*

The general terminology employed for *T.annulata* is that described by Irvin (1987).

Tunisian stocks have been referred to as *T.annulata* Tunisia (*TaTu*) followed by the stock number. The latter was based on the chronology of isolation and the area from which the stock was derived.

Isolates from the same farms had the same stock number and then successive alphabetic letters following this. For example three stocks were isolated from farm 14, they were referred to as *TaTu* 14A, *TaTu* 14B and *TaTu* 14C.

When experimental infections were made, the animal number always followed the stock number. For example stock *TaTu* 11 was used to infect calf 400 *in vivo* and ticks were fed on this calf. The derived parasite material from calf no. 400 was referred to as *TaTu* 11/400. The ticks were used to infect PBM *in vitro*, the PBM were from a naive calf no. 260. The derived cell line was therefore referred to as *TaTu* 11/400/260.

Five cloned macroschizont-infected cell lines, clones (1-5), were used to infect calves. Cell lines derived from these animals were referred to by the animals number preceded by the letter C (for calf) (e.g. *TaTu* C320). All the cell lines set up *in vitro* from the ticks fed on these calves were made using PBM from calf no. 260. They are therefore referred to as, for example, *TaTu* 320/260.

Individual ticks from the batch fed on calf no. 320 were used to infect PBM of calf no. 260. They were referred to as C1, C2 and C3.

## **2.3 EXPERIMENTAL DESIGN AND RESULTS**

### **2.3.1 Cattle**

#### **2.3.1.1 Experimental cattle**

Seven calves were infected experimentally at the ENMV with field isolates selected for vaccine attenuation. Another ten calves were infected experimentally at the IPT. Nine received cloned infected cell lines while the 10th calf was injected with a field isolate.

None of the calves was treated and only the nine calves infected with cloned cell lines, kept at the IPT, were challenged for the purpose of this study. None of these calves suffered severe reactions following challenge.

The details of the calves' infections and the parasite material derived from them and used in the study are given in the relevant chapters.

#### **2.3.1.2 Field cattle**

Eighty-six cattle undergoing clinical theileriosis from different parts of the country were used, from which parasites material was collected. Their sex and age, when known, are given in Table 2.1.

### **2.3.2 Ticks**

#### **2.3.2.1 Laboratory ticks**

*H.a.excavatum* nymphs were fed on the ten calves kept at the IPT. Adults were dissected and infection rates and mean numbers of infected acini assessed.

Salivary gland lysates were prepared from ticks fed on some of these calves. (The details are given in Chapters 6 and 7).

#### **2.3.2.2 Field ticks**

*H.detrutum* field ticks collected from the Chergui Farm (Farm no. 23) and the Bechir Farm were dissected, the infection rates assessed and lysates prepared from salivary glands.

### **2.3.3 *T.annulata*-infected cell lines**

#### **2.3.3.1 Generated from PBM from infected animals**

##### **a) Experimentally infected animals**

Cell lines were established from PBM of all seven calves kept at the ENMV.

Nine cell lines were established from PBM of the ten calves, kept at the IPT.

##### **b) Field clinical cases**

From the 86 blood samples collected from field clinical cases, 14 suffered from transport delays or exposure to excessive heat and were discarded. Twenty-three of the



**Table 2.1      Age and sex of animals from which field isolates were obtained**

Sex	Number of animals
F	21
M	14
not known	16
Age in months	Number of animals
<6	2
6-24	16
24-0	9
>60	5
not known	19
	Total 51

remaining 72 did not transform during cell culture and one was contaminated, with *Trypanosoma theileri*. Forty-eight resulting cell lines were thus obtained from different zones in Tunisia (Table 2.2).

Characteristics of these field samples are given in Table 2.2. Depending on the stage and severity of infection in the animal from which PBM were isolated, schizonts were sometimes observed in PBM, at the other times the first macroschizonts were only detected after several days. In general, cell lines were usually established within 15 days. If no schizonts are detected during that period, the flask is discarded and the sample considered as negative.

#### **2.2.3.2 Generated by *in vitro* infection**

##### **a) From laboratory ticks**

Nine cell lines were established from infection *in vitro* with the batches of *H.a.excavatum* ticks fed on the ten calves, kept at the IPT.

##### **b) From field ticks**

Two cell lines were established from *H.detrutum* ticks collected from the Chergui and Bechir farms.

#### **2.3.3.3 Results of cloning**

The clones established by limit dilution at 0.3 cell per well of schizont-infected cells were generally detected at day 6 after cloning and transferred to 24 well plates between day 8 and 12. Some clones failed to survive passage from the 96 well or 24 well plates necessitating repeated attempts to grow the clones from the original wells. Sometimes it was more successful to passage the cells in only 5mls in the 25cm<sup>2</sup> flask to increase the cell concentration.

The number of successful clones obtained per plate depended on individual cell lines clonability. In theory 30 clones should grow out of 100 wells plated at 0.3 cells per well. In this study a maximum of 25 clones per 100 wells was permitted indicating that the limit dilution had been accurate. If more than 25 clones grew the cell line was recloned. From some cell lines it was not possible to obtain any clones at a dilution of 0.3 cells per well. In other as

**Table 2.2      Characteristic of field isolates**

	Number of field samples	Piropasms in blood smears	Schizonts in Ficoll hypaque PBM	Cell line established
	38	+	+	+
	16	-	-	-
	03	-	+	+
	03	-	-	+
	07	+	-	-
	01	+	+	-
	04	+	-	+
Total	72	50	42	48

few as 2 or 3 clones were obtained. Repeated cloning, or an increase in the number of plates set up for a cell line, increased the cloning success for cell lines which were proving difficult.

#### **2.3.4 Piroplasm**

##### **2.3.4.1 From laboratory cases**

Only three calves infected experimentally (029, 170 and 400) had parasitemias which permitted the isolation of piroplasms. No piroplasms were isolated from the other calves since none reached parasitemia of 5%.

##### **2.3.4.2 From field cases**

Only 12 of the animals identified as field cases were bled for piroplasm collection. The parasitemias recorded in these animals were above 30%. From 9 of them the corresponding schizont-infected cell lines were established. The 3 from which only piroplasms were obtained makes the final number of resulting field stocks 51.

#### **2.3.5 Other parasite material used in the study**

Eighteen schizont-infected cell lines from different countries and four derived clones were available at the CTVM, Edinburgh. Sporozoite antigens for IFAT, were provided from the same source.

Four cloned infected cell lines were kindly provided by Dr B.R.Shields, (Veterinary Parasitology, Glasgow).

At the ENMV, six infected cell lines were already isolated. They were kindly provided by Dr M.Darghouth (Parasitology Department, ENMV, Sidi Thabet).

These cell lines will be described as required.

## **2.4 DISCUSSION**

The piroplasm collections were only made from animals on farms surrounding the ENMV, since they had to be processed the same day. While no problems were encountered for collecting the 10ml samples, the collection of 100ml of blood per animal was always very

difficult, since the animals were very valuable. At £500-1000 for an adult dairy cow, the owners were reluctant to allow this amount of blood to be taken. Were it not for the free treatment provided and the help of the local veterinarian this would not have been permitted at all.

The samples were collected during the theileriosis season, which in Tunisia extends from June-August. During the peak season, in July, several cases were diagnosed each day at the ENMV. Preference for parasite collection was given to animals with both high parasitemia and PCV. Collections of these samples could not have been delayed since delay in treatment is very risky and any fault attributed to the veterinarian would have undermined the confidence of the farmers who were difficult enough to deal with.

This project did not have any official support in Tunisia. Achievements, such as they were, were to a great extent, due to the help of people already working very hard during the theileriosis season which is also the busiest time of the farming year.

A meeting on tropical theileriosis had been organised at the ENMV on the 8th of May 1991. Government veterinarians were invited and preliminary results on current work and findings on theileriosis in Tunisia were presented in order to motivate them in the collection of samples. At the same time 10ml vacutainers, with lithium heparin, were distributed with needles and holders. In response to this initiative samples from all over the country were received during the months following.

In conclusion, the parasite material collected for this study was very diverse. Different life cycle stages of the parasite were included, field isolates as well as experimental isolates were made, both vertebrate and invertebrate *T.annulata* hosts were used as a source of parasite material, and samples were collected from all over the country from sites in different bioclimatic zones (see Chapter 6).

This varied source and character of sample was necessary to assess the extent of diversity amongst isolates of *T.annulata*.

The stocks collected were further characterised by studies at the immunological, the biochemical and the genomic level. Although the biochemical technology was already available

(Melrose, 1983) immunological and genomic studies needed the generation of adequate tools prior to the characterisation of the stocks.

A description of specific materials and methods used and characteristics of the tools produced are dealt with in the next chapters.



## ***CHAPTER THREE***

### ***MONOCLONAL ANTIBODIES DETECTING ANTIGENIC DIVERSITY***

### 3.1 INTRODUCTION

Hybridoma technology has brought two advantages described by Milstein *et al* (1979), namely, the unlimited supply of antibody of predefined specificity and the possibility of using non-purified antigens but obtaining MAbs specific to single antigens. The technique has been invaluable in many areas of biology, such as studies of histocompatibility and differentiation antigens; tumour-associated antigens; T and B cell repertoires and the definition of single antigenic determinants on a wide range of proteins, nucleic acids and sugars. The application of the hybridoma technology to studying micro-organisms such as viruses, bacteria and parasites has permitted the characterisation and separation of subpopulations, the differentiation between developmental stages and the identification of protective antigens. This has resulted in a more accurate identification of micro-organism for diagnosis and epidemiology and the identification of candidate antigens for vaccine production (Yelton, *et al*, 1980).

Parasites are large organisms some of which have complicated life cycles through vertebrate and invertebrate hosts, and their complex antigenic constitution has complicated the isolation of antigens with potential use in protection, diagnosis and characterisation. The production of MAbs raised against these organisms had contributed towards the resolution of these complications.

MAbs have been raised against a wide range of parasites, particularly the protozoa and have been used to define putative protective antigens both *in vivo* (*Plasmodium yoelii* and *P.falciparum*) and *in vitro* (*P.knowlesi*), while others have been used for diagnostic characterisation (Cohen, 1982).

MAbs against *T.annulata* were first raised by Pearson *et al* (1980) to develop an assay for *Theileria*-infected cells. They were used by Pinder and Hewett (1980), Minami *et al*, (1983) and Shiels *et al*, (1986a) in the identification of *Theileria* strains. A potential protective antigen has also been identified by a MAb in *T.annulata* (Williamson *et al*, 1989) and in *T.parva* (Musoke *et al*, 1992).

The aim of the present study was to identify and analyse antigenic diversity amongst Tunisian stocks of *T.annulata* using a panel of MAbs raised to schizont-infected cells. Such

a study would define the diversity at different levels by analysis of stocks from different geographical regions, different sites of isolation, different farms within the the same site and different animals on the same farm. It would also assess the presence of multiple phenotypic variants and or specific ones in defined areas which would have important implications in the selection of cell lines for use in a Tunisian attenuated vaccine. As the parasite is easily maintained as schizont-infected cell lines, this stage was used to raise MAbs as a tool for strain identification (Irvin, 1987). This chapter describes the production and selection of MAbs used to analyse antigenic diversity amongst Tunisian *T.annulata* stocks.

## **3.2 MATERIALS AND METHODS**

### **3.2.1 *Monoclonal antibody production***

#### **3.2.1.1 Mouse immunisation with a schizont-infected cell line**

The first Tunisian isolate of this series of *T.annulata*, *TaTu* 1 (Darghouth, 1992) was used to infect calf 270 and the derived cell line *TaTu* 1/270 (kindly provided by Dr Darghouth, ENMV, Tunisia) used to immunise a Balb/c mouse. The mouse was injected intraperitoneally with  $10^7$  cells from a two day old growing culture of *TaTu* 1/270 and boosted once a month for six months. Bleeding was carried out at regular intervals and the sera adsorbed on PBM from naive cattle. Sera were then monitored for the presence of anti-schizont antibodies using IFAT (see section 3.2.2.1). The last boost was performed three days before the planned fusion.

#### **3.2.1.2 Fusion protocol**

The principle of MAb production is based on fusion between myeloma cells and stimulated splenic lymphocytes (Köhler and Milstein, 1975). Mutant myeloma cells deficient in hypoxanthine-guanine phosphoribosyl transferase (HGPRT<sup>-</sup>) are used in order to allow selection of hybridomas to be carried out. Hybrids of HGPRT<sup>-</sup> and HGPRT<sup>+</sup> cells would be HGRT<sup>+</sup> and will survive in selective medium (Littlefield, 1964). In the case of this study a non-secreting myeloma cell line, Sp2/0-Ag14 (Schulman *et al*, 1978) was used to enable the

production of immunoglobulins (Ig) to be exclusively derived from hybridomas. The selective medium used included azaserine, an analogue of glutamine, and hypoxanthine (both from Sigma).

The MAbs were prepared by fusing the Sp2/0 cells with spleen cells derived from the immunised mouse using polyethylene glycol (PEG) as fusion agent (Davidson and Gerald, 1976). The spleen was teased out and perfused (using an 18 gauge needle), with RPMI 1640 containing antibiotics, at several sites by forcing the medium into the spleen until most of the cells were removed. These were then centrifuged at 300 x g and the red cells lysed by adding 1ml of 0.9%  $\text{NH}_4\text{Cl}$  to the pellet while mixing. After three to five min, the cells were resuspended in serum free medium and centrifuged. The Sp2/0 cells were also washed in serum free medium and both sets of cells were counted. They were then mixed at 5:1 ratio of splenocytes to myeloma cells in serum free medium and centrifuged. In this study,  $3 \times 10^8$  splenocytes were recovered from the spleen and were mixed with  $6 \times 10^7$  myeloma cells. One ml of 50% prewarmed PEG 1000 (Sigma) in serum free medium was added drop wise to the mixed cell pellet over one min. Ten ml of prewarmed serum free medium were then added over 10 min (1ml/min), followed by gentle addition of 30ml of the same medium. The cells were then centrifuged and resuspended in complete medium containing  $5 \times 10^{-5}\text{M}$  hypoxanthine and  $10^{-5}\text{M}$  azaserine (selective medium). They were distributed in 96 well flat bottomed plates, at  $2 \times 10^5$  cells per well in 200ul of medium. The plates were incubated at 37°C in a 5%  $\text{CO}_2$  humidified atmosphere. The selective medium was changed once a week, over a two week period and was then replaced by complete medium when transferring the growing cells to 2ml wells of 24 well plates. The medium was changed by aspirating the liquid through a sterile needle and syringe connected to a pump. The needle was transferred rapidly from one well to the next.

### **3.2.1.3 Selection of positive hybridoma cells**

The supernatants from grown hybridoma cells were screened using IFAT on the infected cell line *TaTu* 1/270. Positive wells producing supernatants reacting strongly with the intracellular parasite together with a number of supernatants also reacting with host cell

determinants, were selected, expanded and cryopreserved as described for *Theileria*-infected cells, while the supernatants were retained and stored at -20°C. Hybridoma cell lines were cloned by limiting dilution at 0.3 cells per well and positive clones used to produce ascites.

#### **3.2.1.4 Ascites production**

This was conducted after Brodeur *et al* (1984). Balb/c mice, were injected intraperitoneally with 0.5ml of Pristane (Sigma) nine days before receiving  $2 \times 10^6$  cloned hybridoma cells. Tapping of the ascitic fluid commenced 7-10 days later. Aliquots of ascites representing each clone were pooled, centrifuged at 800 x g for 15 min at room temperature and the supernatant (with 0.1% azide) stored at -20°C.

#### **3.2.1.5 Immunoglobulin class of monoclonal antibodies.**

Two techniques, Ouchterlony (Ouchterlony and Nilson, 1973) and the Enzyme Linked Immuno Sorbent Assay (ELISA), were used to determine the Ig class of the MAb. Both assays were performed with the same kit (Sigma) using the manufacture's protocol.

#### **3.2.1.6 Terminology used to define the monoclonal antibodies**

Growing cell lines were named after plate's numbers (1-21), followed by alphabetical order and number of the well, according to the plate design. Recovered clones and their ascites were named after the parental hybridoma cell line. For example MAb 7E7 is a clone derived from a parental hybridoma cell line first detected in plate 7, row E, well number 7.

### **3.2.2 Reactivity of monoclonal antibodies**

#### **3.2.2.1 The indirect fluorescent antibody test (IFAT)**

The method used was described by Burridge and Kimber (1972) using schizont-infected cells to detect antibodies in sera from *T.parva* infected cattle. The same approach was used to select and describe MAbs raised against *Theileria* schizonts (Minami *et al*, 1983; Shiels *et al*, 1986a), piroplasms (Glascodine *et al*, 1990) and sporozoites (Williamson, 1988).

Schizont-infected cell lines, piroplasms and sporozoites were used to monitor the reactivity of the MAbs. A bovine lymphosarcoma cell line, BL-20 (Morzaria *et al*, 1984) and PBM isolated from naive calves were used as negative controls.

Schizont-infected cells, normal PBM or BL-20 cells were washed twice in cold PBS at 200 x g for 10 min at 4°C. The volume of the pellet was determined and they were resuspended in three volume of PBS with 1% BPA. The cells were then distributed onto Multispot slides (Flow) by adding one drop to each well and sucking off the liquid. The slides were air dried, fixed in acetone for 15 min and then dried. They were wrapped and stored at -20°C.

The piroplasms were purified as described in chapter two (Section 2.2.6). Blood was taken from *T.annulata* infected animals with a parasitemia of over 30% to allow an easy detection of the MAb reactivity against the piroplasms. The infected erythrocytes were washed twice in cold PBS with 1% BPA then two volumes of PBS/PBA were added to the pellet and the cells were distributed, fixed and stored as described above.

Percoll purified sporozoites (Williamson, 1988), were washed in PBS at 1500 x g for 30 min at 4°C to remove the Percoll. They were resuspended in PBS 1% BPA at 1ml per 64 t.e. and distributed, fixed and stored as described above.

The fixed parasite slides were thawed at 4°C for 30 min then equilibrated at room temperature for 30 min. They were rinsed three times in PBS and drained. Ten  $\mu$ l of hybridoma supernatant, diluted ascites (1/500-1/64000) or serum (1/40-1/640) in PBS were dispensed onto duplicate wells and left to incubate in a humidified chamber for 45 min. The slides were washed three times in PBS and then incubated 45 min with a fluorescein-conjugated goat anti-mouse Ig (Becton-Dickinson) at a dilution of 1/40. After three washes in PBS, the slides were mounted in 90% glycerol in PBS, and examined using a Leitz Dialux 20 microscope fitted with epifluorescence. PBS was used as negative control and polyclonal mouse antiserum to *T.annulata* macroschizonts as a positive control.

### **3.2.2.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting.**



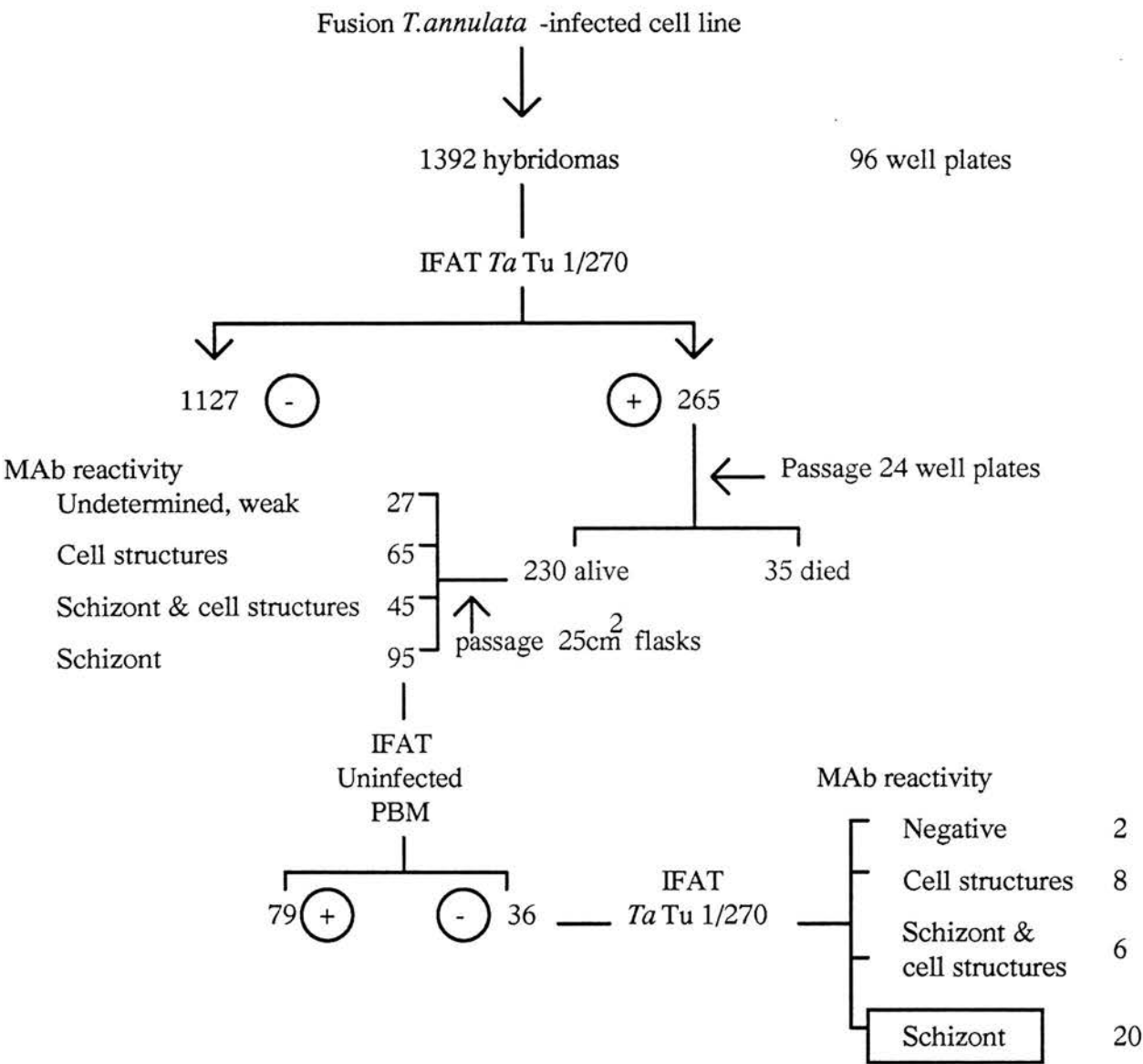
PBM from naive cattle ( $15 \times 10^7$  cells), BL-20 and schizont-infected cells ( $5 \times 10^7$  cells), were washed twice in PBS then lysed by adding 1ml of sample-buffer [62mM Tris HCl, pH 6.8, 2% SDS (w/v), 5% 2-mercaptoethanol (v/v), 7.5% glycerol (v/v)]. The samples were sonicated for  $2 \times 45$ s, boiled for 5 min and centrifuged at  $14,000 \times g$  for 10 min. SDS-PAGE was performed on 8 or 10% polyacrylamide gels according to Laemmli (1970). A 100ul of protein extracts were dispensed per gel track and the resolved proteins were transferred onto nitrocellulose membrane using the technique described by Towbin *et al* (1979). The electroblotting was carried out for 1hr at 120v or 3hr at 30v in transfer buffer (25mM Tris, 192mM glycine and 20% methanol) at 4°C. The membranes were then stained with Ponceau stain (0.3% Ponceau S in 5% trichloroacetic acid) for a few minutes and destained in tap water until the protein bands are visualised. The membranes were blocked, at room temperature, by incubation for two hours in PBS containing 0.5% Tween 20, 5% powdered skimmed milk and 2% normal goat serum (blocking buffer). They were then incubated with diluted ascites (1/500), representing respective MAbs, overnight at 4°C in blocking buffer. After four washes in PBS, the membranes were incubated with peroxidase-conjugated goat anti-mouse immunoglobulins (Jackson Immuno Research Laboratories) in blocking buffer (1/1000) for 1hr at room temperature. The proteins recognised were visualised with 0.05% diaminobenzidine tetra-hydrochloride, 0.01% hydrogen peroxide solution. Molecular weight was determined according to the migration of standard markers (low weight molecular markers, Sigma). Ascites derived from mouse hybridomas secreting anti-human Ig antibodies were used as negative controls (1/500).

### 3.3 RESULTS

#### 3.3.1 Selection of monoclonal antibodies

Growing hybridoma cells were detected from day 6 and were screened between day 9 and day 12. Overall 1392 wells were tested (Fig. 3.1). The supernatants were screened using IFAT on the immunising infected cell line (*TaTu* 1/270) and 265 (16%) reacted strongly with macroschizonts with a number of supernatants also reacting with host cell determinants. The screening at this stage was very rapid and only the reaction against the schizont was

**Figure 3.1:** Results of hybridoma cell line screening for antischizont specificity



considered. The 265 hybridoma cell lines were transferred to 24 well plates; of these 35 did not grow and died. The supernatants of the remaining 230 hybridoma cell lines were rescreened for antiparasite reactions and of these 95 were confirmed as antiparasite, the rest of the positive cell lines reacting as shown in Fig. 3.1. The 230 cell lines were transferred into 25cm<sup>2</sup> flasks and cryopreserved. Given the large number of hybridomas obtained, further investigations were carried out on the hybridoma cell lines showing only antiparasite specificity. Therefore supernatants from the 95 antiparasites hybridomas were tested on uninfected PBM and only 36 were negative. The same supernatants, were retested on *TaTu* 1/270 and the results are shown in Fig. 3.1. In conclusion 20 hybridoma cell lines were retained as parasite specific and cloned by limiting dilution at 0.3 cells per well, positive clones from 10 hybridoma cell lines were obtained. Three cell lines did not produce any clones and from the remaining seven only negative clones were recovered. The 10 hybridoma cell lines were subjected to a second cloning in the same condition and clones which produced the highest titre of antibodies were cryopreserved. Of these one clone was used to produce ascites which was used for the characterisation of *Theileria*-infected cells in this study, the respective MAbs being named after their parental hybridoma cell lines (Table 3.1).

A further screening of the 10 MAbs listed in table 3.1 was carried out using the ascites dilutions on both *TaTu* 1/270 and uninfected PBM antigens by IFAT. Nine MAbs (2A9, 3D10, 7E7, 11H5, 12D5, 12H8, 17H2, 19D6 and 19E11) conserved their specificity against the schizont and did not react with uninfected PBM whereas one MAb, 19B7, showed in addition to its reactivity with the schizont, a doubtful reactivity with uninfected PBM. The proportion of cells showing fluorescent schizonts correlated with the percentage of parasitised cells as seen on Giemsa stained cystospin smears. The working dilutions of the MAb ascites and their Ig subclasses were determined and are described in table 3.1.

### 3.3.2 Analysis of antigenic diversity by immunofluorescence

The ten MAbs were then tested on a panel of nine schizont-infected cell lines: *TaTu* 2, 3, 4, 5, 6 (Darghouth, 1992) and *TaTu* 2/029, 9A, 9B and 10. They were also tested on

**Table 3.1:** Limiting dilutions of MAb ascies as determined by the IFAT on *TaTu* 1/270 antigen.

MAbs	Ig subclass	Ascites dilutions								
		1/500	1/1000	1/2000	1/4000	1/8000	1/16000	1/32000	1/64000	1/128000
12H8	IgG3	□◇	∅	∅	•					
19B7	IgM	○	○◇	∅	∅	•				
19D6	IgG3	○	○◇		∅	•				
2A9	IgM	○	○	○◇	∅	•				
11H5	IgM	○	○	○◇	□	∅	•			
17H2	IgG2a	○	○	○◇	□	∅	•			
12D5	IgG2a	○	○	○	○◇	∅	•			
3D10	IgG1	○	○	○	○◇	□	∅	•		
19E11	IgG1	○	○	○	○	○	○◇	□	•	
* 7E7	IgG1	○	○	○	○	○	○	○◇	○	□

○ Strong positive

◻ Positive

∅ Weak positive

◇ Dilutions used for IFAT

• Negative

\* Very bright

piroplasms antigens of *TaTu 8* and *Ta Ankara* (Schein *et al* , 1975) and sporozoite antigens of *Ta Ankara* (kindly provided by Mrs L. Bell-Sakyi, CTVM, Edinburgh).

Eight MAbs, 2A9, 3D10, 11H5, 12D5, 17H2, 19B7, 19D6 and 19E11 reacted similarly with all stocks, the fluorescence being limited to the macroschizont, MAbs 19B7 and 19D6, however, showed a different staining profile as the fluorescence diffused from the macroschizont into the cytoplasm.

MAbs 7E7 and 12H8, displayed a variable reactivity with the nine stocks. MAb 7E7 reacted with a small proportion of the cells (1-2%) in two stocks, was unreactive with three and reacted totally, as with the immunising cell line, with four stocks (Table 3.2). MAb 12H8 reacted positively only with *TaTu 2* by staining 50% of the infected cells (Table 3.2).

In order to determine the stage specificity of these MAbs they were tested against the sporozoite and piroplasm stages of *T.annulata*. Four MAbs were positive on other parasitic stages. MAbs 3D10 and 19E11 both reacted with sporozoites whereas MAbs 12D5 and 17H2 reacted with piroplasms. None of the 10 MAbs reacted with all the three parasite stages.

To determine whether the reactivity of the MAbs remain stable after cloning of macroschizont-infected cell lines and whether the variable reactivity detected by MAbs 7E7 and 12H8 within some stocks, showing both positive and negative cells was due to a mixture of subpopulations presenting different phenotypes, 14 cloned infected cells, derived from stocks *TaTu 2* and *TaTu 5* were tested. All clones presented similar pattern of reactivity. They showed a negative reactivity when tested with MAbs 7E7 and 12H8 and were totally positive (90-100% of the cells stained) with the remaining MAbs. It was suspected that the antigenic diversity showed by the former MAbs amongst *Theileria*-infected cells might be due to the presence of mixed negative and positive subpopulations with respect to these MAbs. This was later confirmed for MAb 7E7, when clones from *T.annulata* Ankara, in which 50% of the cells were stained with this MAb, segregated either as totally negative clones or positive clones. MAb 12H8 did not react with *T.annulata* Ankara, therefore no attempts were made to test this MAb with the above mentioned clones.

**Table 3.2** Reactivity of MAbs 7E7 and 12H8 on Tunisian stocks of *T.annulata*-infected cell lines using IFAT

<i>TaTu</i>	Site of Isolation	7E7	12H8
1/270	1	++	+
2	1	.*	(+)
2/029	1	-	-
6	3	++	-
9A	3	++	-
9B	3	++	-
10	3	-	-
3	12	++	-
5	16	.*	-
4	17	-	-

-	0%	of the cells stained
.*	1-2%	of the cells stained with a strong signal
(+)	50%	of the cells stained with a weak signal
+	95-100%	of the cells stained
++	95-100%	of the cells stained with a strong signal



In conclusion two MAbs, 7E7 and 12H8, out of ten tested by IFAT against a panel of macroschizont infected cells, detected antigenic variability amongst these *T.annulata* infected cells. This variability was shown to be due to the presence of two subpopulations according to the MAb 7E7 reactivity. These being found either as single entities or could coexist within one isolate (this was also confirmed from the data obtained from the remaining Tunisian stocks tested and presented in Chapter 6, see Section 6.2.1.1 and Fig. 6.2).

### ***3.3.3 Characterisation of the polypeptides detected by the monoclonal antibodies by Western blotting.***

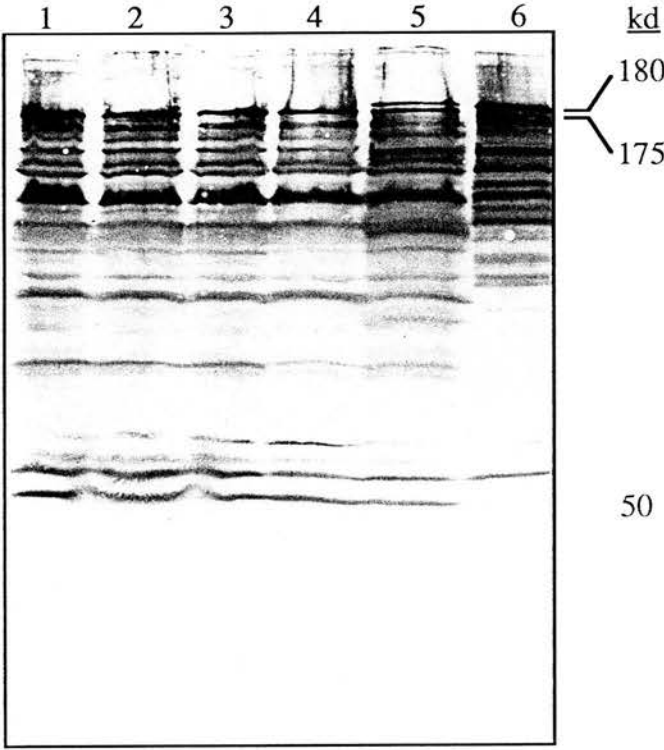
TaTu 1/270 antigen extracts were separated by SDS-PAGE and Western blotted. They were then probed with the ten MAbs, together with control ascites, in order to determine their respective reactive proteins. Six MAbs recognised specific proteins of variable molecular weight while the remaining four, 2A9, 11H5, 12H8 and 19B6 did not show any reaction. MAbs 12D5 and 17H2 showed a variable strength of reaction to proteins ranged between 50 and 180kDa. They both, however, strongly stained the 180kDa protein (major band). The two MAbs showed the same profile which is illustrated by MAb 12D5 profile in Fig. 3.2. MAbs 3D10 and 19E11 detected a protein of the same molecular weight of 148kDa, illustrated by the reactivity of MAb 3D10 in Fig. 3.3 (Track 8). MAb 7E7 detected a protein of 38kDa (Fig. 3.4, track 4).

Uninfected PBM and BL-20 antigen extracts were used as controls. The above mentioned MAbs did not have any reactions against these extracts. The reactivity of MAbs 12D5 and 3D10 on an extract of BL-20 is shown below in Figs 3.6 (Track 6) and 3.7 (Track 5).

MAb 19B7 detected several proteins with molecular weights ranged from 50 to 180kDa (Fig. 3.5). This MAb also stained proteins in antigen extracts of uninfected PBM and BL-20. It reacted strongly with a 50kDa protein and stained weakly a doublet of approximately 30kDa in antigen extracts of uninfected PBM (Fig. 3.5, track 1). The reactivity of this MAb against BL-20 antigen extract was similar to the reactivity detected in *T.annulata* infected cells (data not shown).

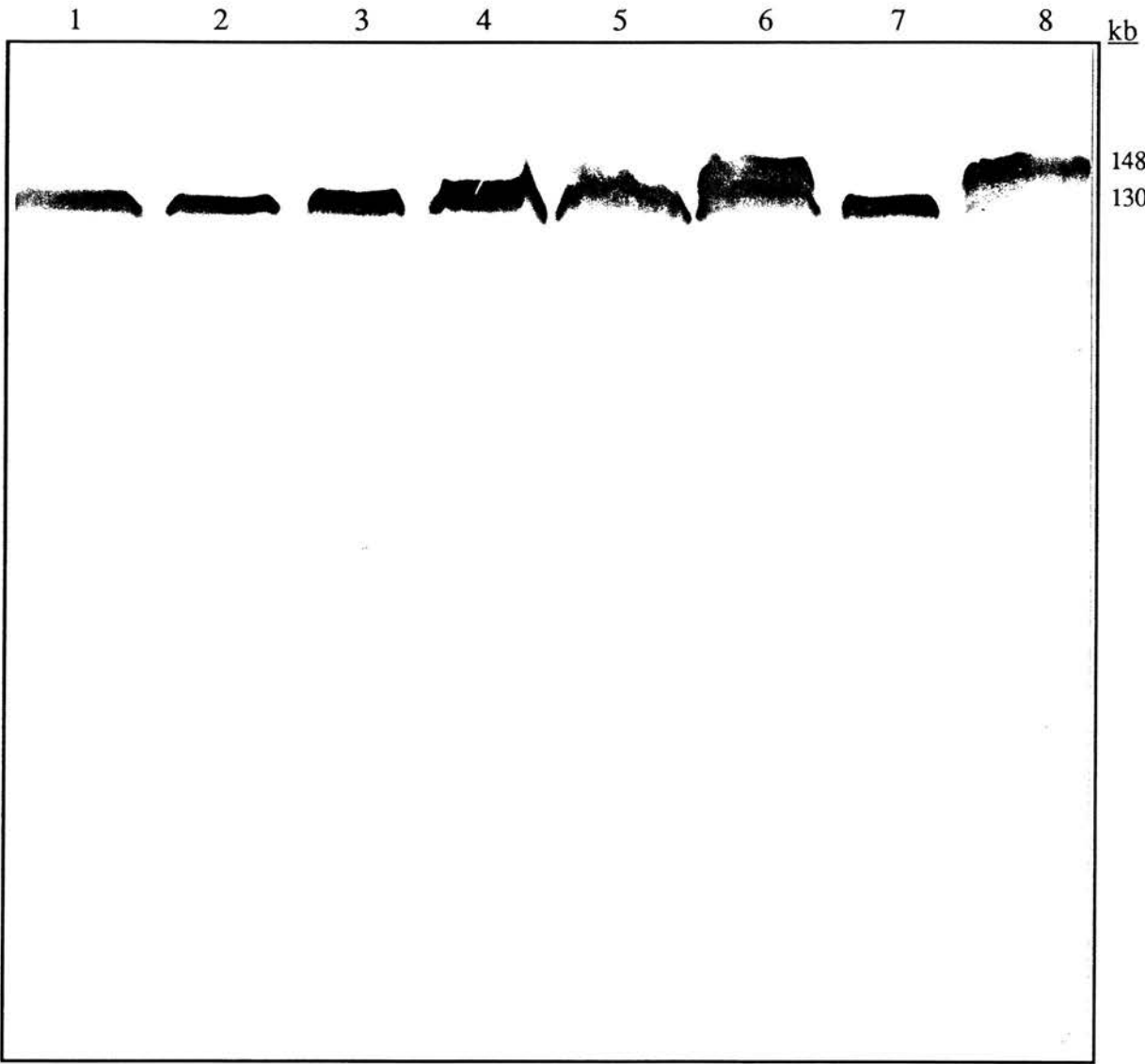
**Figure 3.2** Immunoblot analysis of antigens in different stocks of *T.annulata*-infected cell lines recognised by MAb 12D5. Tracks correspond to stocks.

Tracks	<i>T.annulata</i> -infected cell lines
1	TaTu 5
2	TaTu 4
3	TaTu 3
4	TaTu 2/029
5	TaTu 2
6	TaTu 1/270



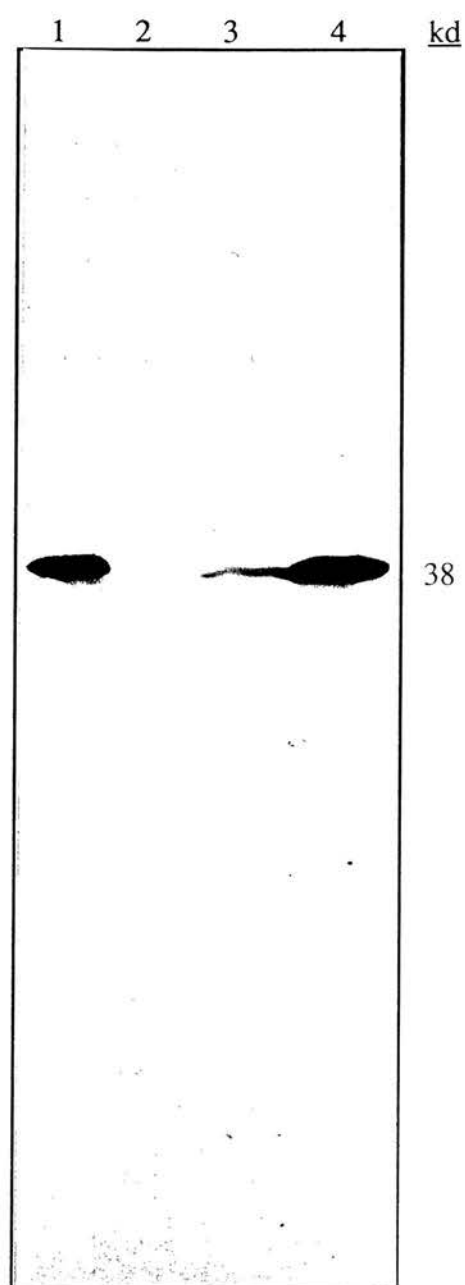
**Figure 3.3** Immunoblot analysis of antigens in different stocks of *T.annulata*-infected cell lines recognised by MAb 3D10. Tracks correspond to stocks.

Tracks	<i>T.annulata</i> -infected cell lines
1	<i>TaTu</i> 5
2	<i>TaTu</i> 4
3	<i>TaTu</i> 3
4	<i>TaTu</i> 10
5	<i>TaTu</i> 9B
6	<i>TaTu</i> 9A
7	<i>TaTu</i> 6
8	<i>TaTu</i> 1/270



**Figure 3.4** Immunoblot analysis of antigens in different stocks of *T.annulata*-infected cell lines recognised by MAb 7E7. Tracks correspond to stocks.

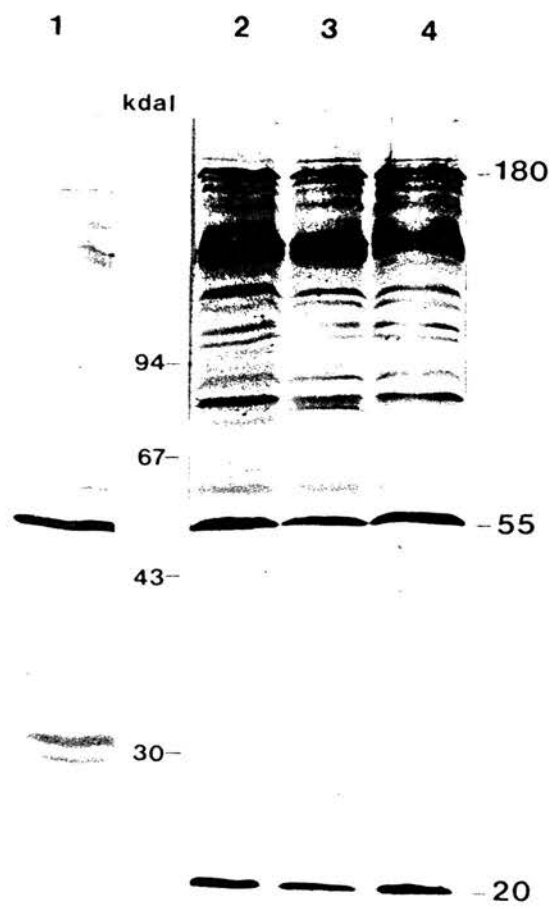
Tracks	<i>T.annulata</i> -infected cell lines
1	TaTu 3
2	TaTu 4
3	TaTu 5
4	TaTu 1/270





**Figure 3.5** Immunoblot analysis of antigens in different stocks of *T.annulata*-infected c lines and normal PBM recognised by MAb 19B7. Tracks correspond stocks.

Tracks	Stocks
1	Uninfected PBM
2	TaTu 1/270
3	TaTu 2
4	TaTu 9A



To further analyse the reactivity of the six MAbs, these, together with control ascites, were tested with different schizont-infected cell line extract antigens.

MAbs 12D5 and 17H2 showed differences in both the major band and the remaining bands (minor bands) detected. The reactivity of MAb 12D5 is illustrated in Fig. 3.2. A different major protein of 175kDa was detected in the nine stock listed in Table 3.2. From these *TaTu* 2 (Fig. 3.2, track 5) presented both the 175kDa and 180kDa proteins. The observed profile of the minor bands was similar for the extracts derived from stocks *TaTu* 5, 4, 3, 2/29 and *TaTu* 2 (Fig. 3.2, track 1-5), but varied slightly from the immunising cell line *TaTu* 270/1 (Fig. 3.2, track 6). MAb 17H2 presented exactly the same pattern of reactivity on these cell lines (data not shown).

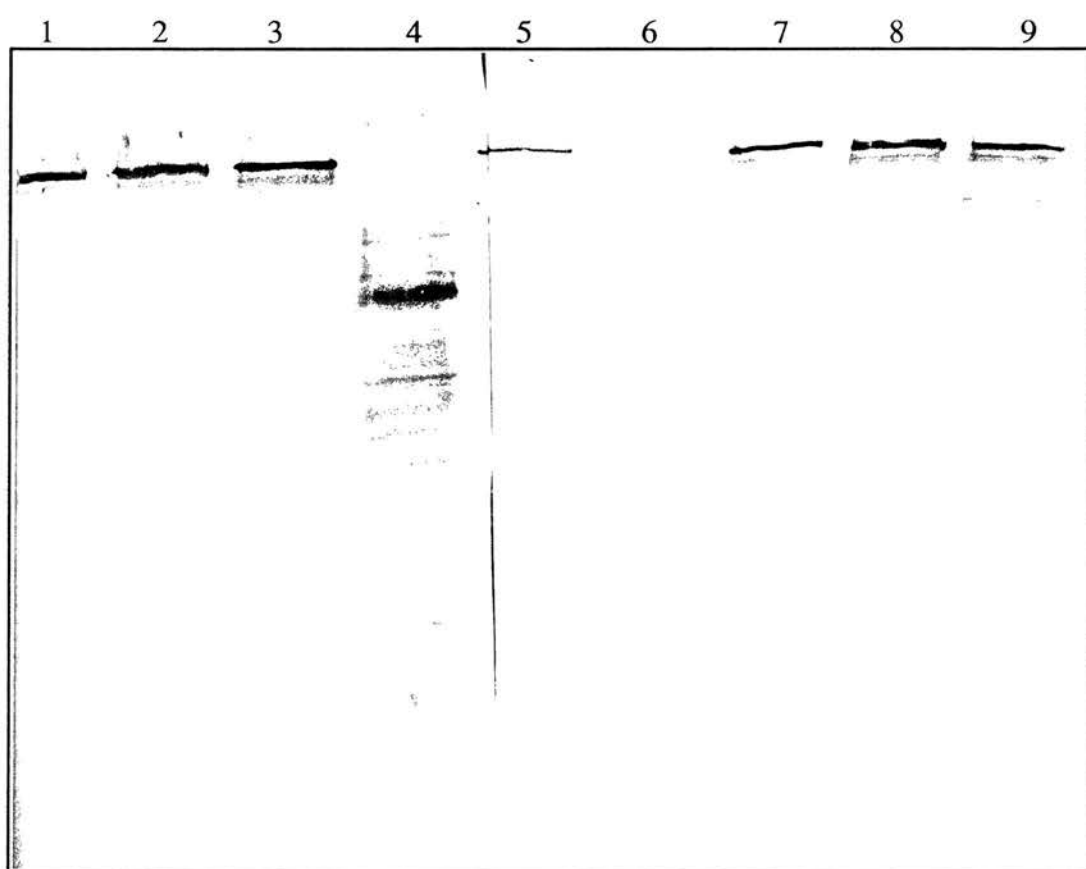
MAbs 3D10 and 19E11 detected a 130kDa protein in all nine stocks tested, but only *TaTu* 9A antigen extract showed the presence of both the 130kDa and 148kDa proteins. The latter being the protein originally detected in extract from the immunising cell line *TaTu* 1/270. The reactivity of MAb 3D10 is illustrated by Fig. 3.3.

The separation of the two sets of proteins detected by these four MAb, was not always seen. To investigate this problem further lower concentration of the samples (1/10 of the initial sample) and of the acrylamide gels (8%) were tested (Figs. 3.6 and 3.7). None of the modifications mentioned above helped in a better resolution of the proteins as seen in the Figs. 3.6 and 3.7.

In order to investigate further the presence of two proteins in protein extracts of stocks *TaTu* 2 and *TaTu* 9A when respectively tested with MAbs 12D5/17H2 and 3D10/19E11, six cloned cell lines deriving from each of these stocks were analysed. The six cloned infected cell lines from each stock showed a similar pattern. MAb 12D5/17H2 stained only the major band of 175kDa in antigen extracts of *TaTu* 2 clones whereas the minor bands for these clones were similar to those seen in the parental stock. The reactivity of MAb 12D5 is illustrated by Fig. 3.8A. MAbs 3D10/19E11 detected only a 130kDa protein in antigen extracts of *TaTu* 9A clones. The reactivity of MAb 3D10 is illustrated by Fig. 3.8B. The results showed that the presence of the doublet of proteins described above corresponds to the presence of mixed subpopulations within the same stock, which could be separated by

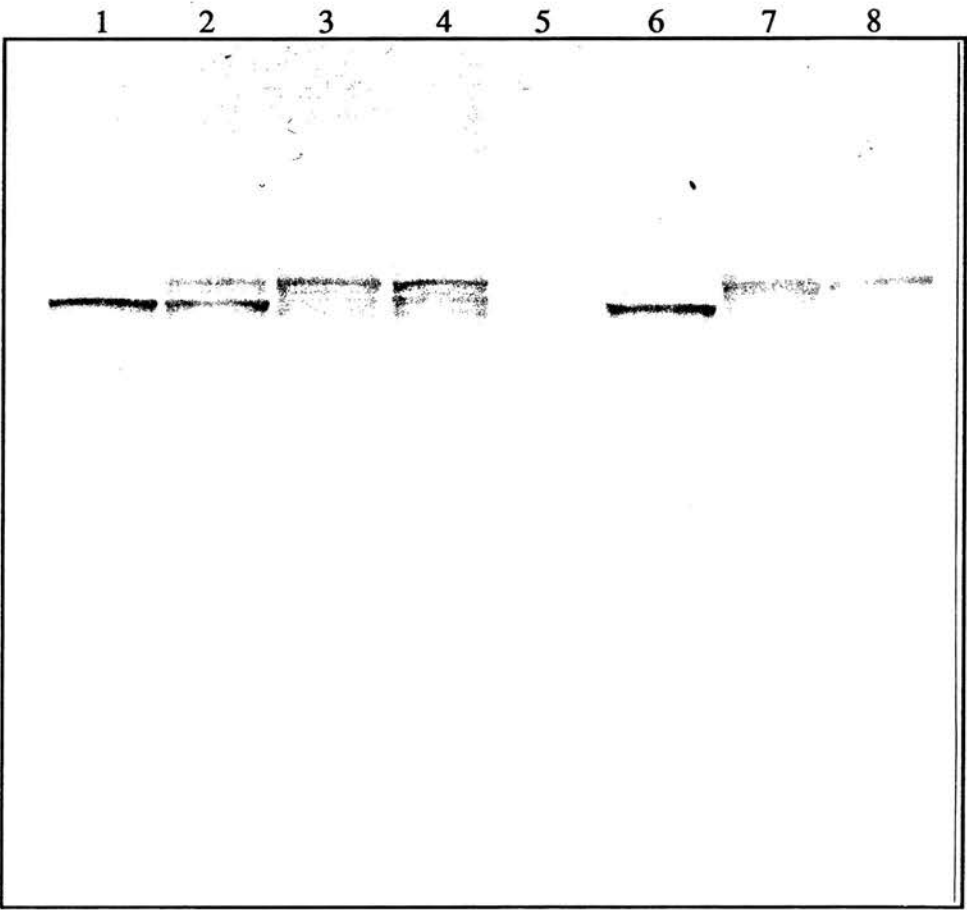
**Figure 3.6** Immunoblot analysis of antigens in different stocks of *T.annulata*-infected cells, *T.annulata* piroplasms, and BL-20 recognised by MAb 12D5. Tracks correspond to stocks.

Tracks	Stocks
1 and 7	<i>TaTu</i> 9A cell line
2 and 8	<i>TaTu</i> 2 cell line
3 and 9	<i>TaTu</i> 1/270 cell line
4	<i>TaTu</i> piroplasms
5	<i>TaTu</i> 9A cell line (1/10 diluted sample)
6	BL-20



**Figure 3.7** Immunoblot analysis of antigens in different stocks of *T.annulata*-infected cells and BL-20 recognised by MAb 3D10. Tracks correspond to stocks.

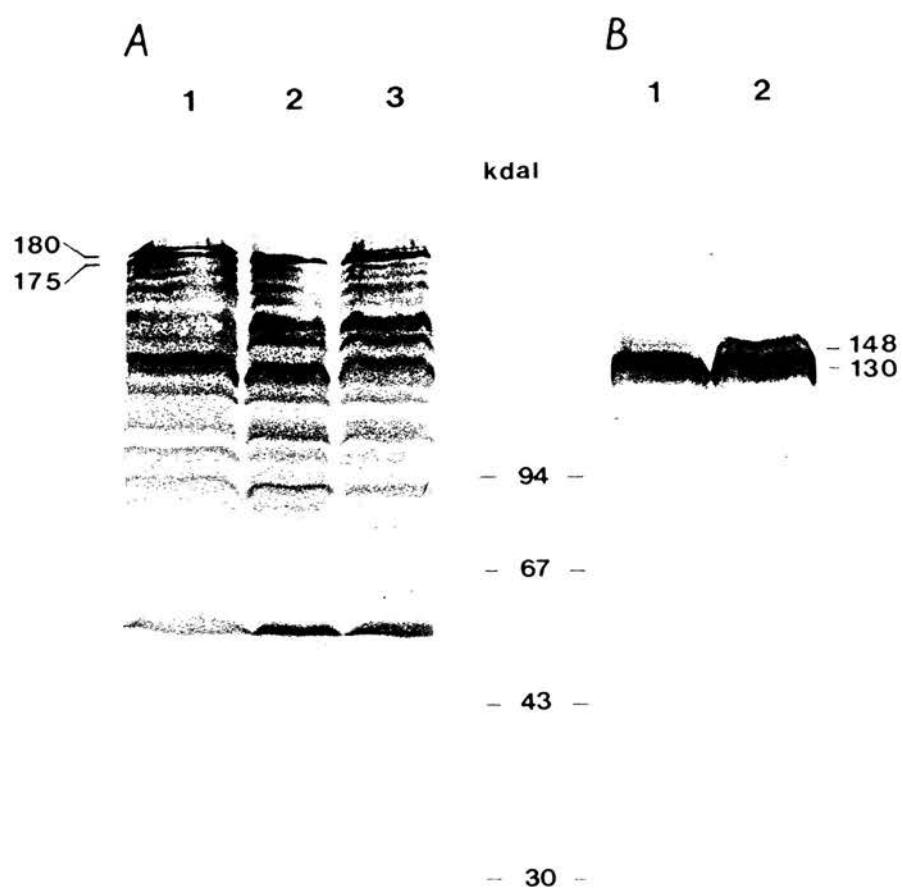
Tracks	Stocks
1 and 6	<i>Ta</i> Tu 4 (1/10 dilution sample)
2 and 7	<i>Ta</i> Tu 9A (1/10 dilution sample)
3 and 8	<i>Ta</i> Tu 1/270 (1/10 dilution sample)
4	<i>Ta</i> Ankara
5	BL-20





**Figure 3.8** Immunoblot analysis of antigens in different stocks of *T.annulata*-infected cell lines and their clones recognised by MAbs 12D5 (A) and 3D10 (B). Tracks 1 and 2 correspond to stocks.

	Tracks	<i>T.annulata</i> -infected cell lines
A	1	<i>TaTu 2</i>
	2	<i>TaTu 2</i> clone
	3	<i>TaTu 2</i> clone
B	1	<i>TaTu 9A</i> clone
	2	<i>TaTu 9A</i>



cloning the parental cell line. The data also showed that, although these MAbs did not detect any antigenic diversity amongst the stocks by IFAT, they displayed a variable reactivity when tested against protein extracts from these stocks, by Western blotting. This variability was expressed by the presence of a polymorphic protein detected by each pair of MAbs. These proteins are parasite specific since none of these MAbs reacted with the controls (uninfected PBL and BL-20). Since MAbs 12D5/17H2 reacted by IFAT with piroplasms, antigen extracts from *TaTu* 9A piroplasms were probed with these MAbs. The results presented by MAb 12D5 are illustrated in Fig. 3.6, track 4. Both MAbs showed the same pattern of reactivity consisting of a smear of stained proteins.

MAb 7E7 did not detect any protein in antigen extracts from cell lines which were negative by IFAT. However, it showed a weak reactivity with *TaTu* 5 (Fig. 3.4, track 3) and *TaTu* 2 (data not shown) and reacted similarly to the immunising stock with the remaining cell lines which were known to be positive by IFAT. Its reactivity is illustrated by Fig. 3.4.

MAb 19B7 when tested with different macroschizont-cell line extracts did not detect any difference as compared to the immunising cell line *TaTu* 1/270. The results are illustrated by Fig. 3.5.

Table 3.3 summarises the results obtained with the ten MAbs in the IFAT assays and their reactive proteins characterised by Western blot analysis.

### 3.4 DISCUSSION

Until the advent of hybridoma technology (Köhler and Milstein, 1975, 1976), strain differentiation in *T.annulata* was based on virulence (Sergent *et al*, 1924), geographical location of the isolates and cross immunity trials (Adler and Ellenbogen, 1936; Pipano and Tsur, 1966). MAbs were first raised against *T.parva* schizonts (Pearson *et al* 1980; Pinder and Hewett, 1980), and showed different degrees of reactivity in different stocks, indicating that such differences could be used in strain differentiation (Minami *et al* , 1983). Antigenic diversity using MAbs was first shown in *T.annulata* by Shiels *et al*, (1986a). This study described two MAbs detecting variation between and within 12 stocks from different countries. In the present study MAbs have been raised against schizont-

**Table 3.3** Stage specificity of MAbs as determined by IFAT and proteins recognised by Western blot reactivity against macroschizont-infected cell extracts

Monoclonal antibodies		IFAT			Western blot reactivity	
Designation	Subclass	Sporozoite	Schizont	Piropiasm	Mol. Wt. of proteins detected on <i>T.annulata</i> macroschizont extracts (kDa)	
3D10 19E11	IgG1	+	+	-	148 148/130 130	
12D5 17H2	IgG2a	-	+	+	Major bands 180 180 / 175 175	Minor bands 50-180
2A9 11H5 19D6 12H8	IgM IgM IgG3 IgG3	- - - -	+ + +* x	- - - -	Negative	
7E7	IgG1	-	x	-	38	
19B7	IgM	-	+*	-	Multiple bands	

- + 80-95% of the cells stained  
 x 0-95% of the cells stained (See Table 3.2)  
 \* Additional reactivity detected in the cytoplasm.

infected cell lines to investigate strain differences in *T.annulata* within Tunisia. Few problems were encountered during the production of these MAbs.

The average number of cells recovered from a mouse spleen, which has been stimulated for the production of MAbs is estimated to be  $5-10 \times 10^7$  cells per organ (Kenneth, 1981; Bastin *et al* , 1983). In the case of this study  $3 \times 10^8$  cells were recovered and an unusually large spleen was observed when the abdomen of the mouse was opened. A possibility that *Theileria*-infected cells are highly antigenic in mice should be considered for the phenomenon observed.

A number of hybridoma cells lost their activity upon passage, this could be due to either a lost of the secreting activity of the cells or to the presence of both secreting and non-secreting cells in the wells with the latter growing more successfully in culture.

After the cells were transferred to the 25cm<sup>2</sup> flasks, only 20 out of 36 retained anti-schizont reactivity (Fig. 3.1). One of them, MAb 19B7, reacted very weakly on uninfected PBM and this was not noticed on the first screening. Both MAbs 19B7 and 19D6 had different staining fluorescence patterns from the other MAbs. The fluorescence was undelimited and spread out from the parasite throughout the cytoplasm. This was not due to mixed hybridoma populations reacting with both cytoplasm and parasites since the two MAbs conserved their reactivity after cloning by limiting dilution. It is more likely that these MAbs recognise a protein which might be of host origin but which is concentrated around the intracytoplasmic parasite, rather than being a structural antigen of the latter.

The 20 hybridoma cell lines were cloned by limiting dilution and this was successfully achieved for ten of the lines. These were then recloned to ensure their reactivity.

The first objective in raising the MAbs was to generate reagents which detected antigenic polymorphism and so would be useful in strain differentiation. To extend this analysis molecular characterisation of the antigens detected by these antibodies was also carried out. Ten MAbs were selected and then tested on nine additional schizont-infected cell lines to investigate antigenic diversity.

Using the IFAT assay only two MAbs, 7E7 and 12H8, detected antigenic polymorphism (Table 3.2). MAb 7E7 had a range of reactivity that revealed it to be a good

reagent for assessing parasite diversity. It reacted totally with some of the cell lines, partially with others and was negative on the remaining cell lines. MAb 12H8 displayed a more restricted reactivity, since it only reacted (weakly and partially) with one of the nine infected cell lines studied (Table 3.2).

For further studies using IFAT on a wide range of infected cell lines, MAb 7E7 was selected for the following reasons:

- (i) The intensity of the fluorescence shown. Even if only 1% of the cells were stained this was intense making clear cut assessments possible.
- (ii) The pattern of reactivity displayed.
- (iii) The working dilution of the antibody (Table 3.1).

This MAb was therefore used to study antigenic diversity in the 48 *T.annulata* stocks collected from Tunisia to investigate the antigenic variability within a bioclimatic zone, within a site of isolation and within a farm. The results are presented and discussed in chapter six.

Previous studies on *T.parva* (Minami *et al* , 1983, Conrad *et al* , 1987b) have described the generation of MAbs which detected parasite antigenic diversity within *Theileria* species. These were used in strain identification and in a correlation between the antibody profiles and the ability of infected cell lines to cross protect (Irvin *et al*, 1983). In *T.annulata*, antigenic diversity between isolates from different countries was detected by Shiels *et al* (1986a). That study described two polymorphic MAbs tested on 12 macroschizont-infected cell lines using IFAT. Six others reacted with all stocks. In the present study on *T.annulata*, two MAbs, 7E7 and 12H8 detected variation within and between the isolates and provide evidence for the existence of two different antigenic populations within Tunisia. Using a limited number of cloned macroschizont-infected cells deriving from stock *TaTu* 2, which showed the presence of both negative and positive cells when stained with these MAbs, only the negative population in both cases was isolated. This probably reflected the fact that the negative cells represented a high proportion of the mixed population (98% in the case of MAb 7E7) or were more easily cloned *in vitro*. The latter

might be true for MAb 12H8, since 50% of the cells were stained by this MAb in stock *TaTu* 2. However both populations were obtained from a cloned cell lines of *T.annulata* Ankara, of which 50% of the cells were recognised by MAb 7E7. This might mean that the populations defined by MAb 7E7 do have the same ability to grow *in vitro* when they are present at similar proportions in the parental cell line whereas those defined by MAb 12H8 do not.

The proteins recognised by six of the MAb, selected in this study, were also characterised using Western blotting. The reactivity of MAb 7E7 was similar to its reactivity by IFAT and was shown to recognise a 38kDa protein in extracts derived from cells which were known to be recognised by the antibody using IFAT. MAbs 3D10 and 19E11, which reacted similarly with all stocks by IFAT, showed variation between different stocks by Western blotting. The pattern was indicative of two proteins close in molecular mass (130/148kDa) which could either be detected exclusively within different stocks or together in the extracts derived from a single stock (*TaTu* 9A). The dominant form expressed was the 130kDa in the majority of the cell lines studied. Only the immunising stock, *TaTu* 1/270, expressed the 148kDa protein alone. The dominant form was also observed in all the clones derived from stock *TaTu* 9A which contained both the 130kDa and 148kDa molecules. These results probably reflect that parasite subpopulations expressing the 130kDa protein are more frequent in the *T.annulata* population circulating in Tunisia or that these grow better *in vitro*. Other studies on *Theileria* did not investigate the frequency of different proteins detected in the parasite population, but they showed the presence of proteins which varied in size between the different parasite stocks. Four MAbs recognising the macroschizont of *T.parva* were described by Shapiro *et al* (1987). They reacted with the same antigen which differed in molecular mass in different *T.parva* stocks. In another study on *T.parva*, Toye *et al* (1991) showed the presence of an immunodominant molecule, polymorphic in size (PIM), using MAbs and bovine antisera. Different sizes of PIM expressed in the uncloned parental cell line were reported but cloned cell lines only expressed a single type of molecule. Therefore antigenic diversity, detected in *Theileria* antigen proteins, is a commonly



described phenomenon and could have important implications for cross-protection in immunisation, particularly with respect to recombinant vaccines.

A polymorphic protein in *T.annulata* has also been described by Dickson and Shiels (1993). This protein was detected in *T.annulata* merozoite and piroplasm extracts by both MAbs and immune bovine sera. The molecules described were in the range of 30-32kDa and could either coexist in a single isolate or be present separately in other isolates. Variation was shown between isolates from different countries as well as from isolates within a country. This emphasises the importance of such studies especially for the design of a subunit vaccine, including different components, e.g. polypeptides from different parasite stages, which must protect against all variants of the parasite at least within a defined region. This could be achieved only if the polymorphism presented by such molecules is investigated and correlated with cross-immunisation trials.

A study on the *T.sergenti/orientalis/buffeli* group of parasites, using immune sera, also showed variation between stocks in a molecule in the same range of molecular mass, 33-34kDa (Kawazu *et al* , 1992). The authors suggested that this polymorphic protein could be used as a potential marker to distinguish these *Theileria* species. Therefore antigenic polymorphism in *Theileria* proteins is not only important for vaccine studies but also for taxonomy, which is particularly indeterminate for the *Theileria* isolated from the Far East.

Further studies are needed to clarify the molecular basis of the microheterogeneity revealed by the differential reactivity of the MAbs. These may reflect differential glycosylation of a single protein by different subpopulations, explaining their close apparent molecular mass, as shown for the 30-32kDa proteins in *T.annulata* by Dickson and Shiels (1993) or may be due to differences in the size of the polypeptide.

MAbs 12D5 and 17H2, although reacting in a different way, also detected two forms of a major protein, 175-180kDa, and two minor protein profiles. A possibility that the minor bands detected by these MAbs might represent the same protein which undergoes processing needs to be considered. In this eventuality, the higher molecular weight proteins could be the precursors. This has been observed with *P.knowlesi* (Ellis *et al* , 1983) and *T.annulata* (Williamson, 1988). However, these proteins could be different, while sharing a

common determinant, or epitope causing cross-reactivity (Ghosh and Campbell, 1986). The preparation of the protein extracts for SDS-PAGE used fresh material immediately denatured. The use of protease inhibitors did not change the pattern observed for MAbs 12D5 and 17H2 (data not shown), so it is unlikely that the minor bands observed were due to protease activity.

MAbs 3D10/19E11 were of the same Ig class, IgG1, and this was also the case for MAbs 12D5/17H2, which were both IgG2a. The profile of reactivity being identical for each pair, it is therefore likely that they recognise the same antigens. However additional cross-inhibition experiments would be required to confirm without doubt that the initial hybridoma cell lines were identical.

A very common phenomenon, detected in this study, was first noticed in the early test of the MAbs on the panel of nine macroschizont-infected cell lines. Cell line *TaTu 2* was used to infect calf 029. The derived cell line from this calf was compared using IFAT and Western blotting with the parental cell line. It appeared that, whereas *TaTu 2* had 2% of the cells stained by MAb 7E7 and presented the two forms of the major protein detected by MAbs 12D5/7H2, *TaTu 2/029* was completely negative on testing with MAb 7E7 (Table 3.2) and showed only the lowest molecular weight band of the major protein detected by MAbs 12D5/17H2 (Fig. 3.2). This phenomenon was assumed to be due to selection of a certain type of parasite populations either *in vivo* or during the establishment of the cell line *in vitro*. This is discussed further in chapter six.

The fluorescence shown by MAbs 19B7 and 19D6 was distinct, on the basis of the observation that it was found to extend to the cytoplasm of the host cell, whereas all other MAbs had a very discrete reaction against the macroschizont. MAb 19D6 did not show any reaction with uninfected PBM whereas MAb 19B7 showed an irregular weak reaction on these cells in IFAT. One possibility is that the reactions shown by such MAbs could be due to a cross-reaction with a bovine protein closely involved with the parasite structure.

The reactivity of MAb 19B7 using Western blotting was very interesting. This MAb detected few proteins on extracts from uninfected PBM, mainly a 50kDa protein, and a range of proteins from 50-180kDa on extracts from *T.annulata*-infected cells and the uninfected

lymphoid cell line BL-20. It is possible that the proteins detected might occur at a low level in normal cells but their level is enhanced during transformation in both BL-20 and infected cells.

MAB 19D6 did not detect a polypeptide on Western blotting. It is possible, however, that it may have shown a similar profile to 19B7. The lack of reaction by IFAT with uninfected PBM was probably due, either to a lower affinity to the protein detected, or to the low level of antigenic determinants present in uninfected cells. Further analysis is needed to clarify the nature and role of the proteins detected by these MAbs, especially MAB 19B7. The mechanisms involved in the expression of these proteins could be important to a better understanding of both parasite biology and lymphoblastoid transformation of eukaryotic cells.

Only six of the MAbs described in this chapter were stage specific using IFAT. MAB 3D10 and 19E11 reacted with sporozoites whereas MAB 12D5 and 17H2 reacted with the intraerythrocytic piroplasms. MAB 12D5 and 17H2 were tested, using Western blotting, against piroplasm antigen extracts. They showed a smear that could not be clearly interpreted. The reactivity of MAB 12D5 is illustrated in Fig. 3.6 (Track 4). The profile detected by these MAbs on schizont-infected cell extracts was complex but interpretable to a certain extent (Fig. 3.2). One possibility to explain the differences in profile between the two stages would be the presence of common carbohydrate epitopes recognised by these MAbs which are more abundant in the piroplasms (Dr B.R.Shiels, Personal communication).

None of the 10 MAbs reacted, by IFAT, with all three parasite stages, showing the *T.annulata* antigenic profiles change markedly during the life cycle. However, the cross-reactivity between the sporozoite stage and the schizont stage on one hand and the schizont and the piroplasms on the other hand showed that the changes in antigenicity may be occurring in a step wise fashion. MAbs reacting with *T.parva* PIM (Toye *et al* , 1991) in the schizont stage also reacted with the sporozoite stage of the parasite, whereas Glascodine *et al* (1990) reported, in studies on *T.annulata*, a MAb reactivity with all three parasite stages as well as a MAb reacting with the macroschizont and the piroplasm stages only and a MAb reacting with the sporozoite and the macroschizont stages only. The MAbs described by

Shapiro *et al* (1987), reacting with the macroschizont of *T.parva*, did not show any cross-reactivity with either the sporozoite or piroplasm stages.

Both common and stage specific antigens have been identified in *T.annulata* using bovine immune sera (Kachani *et al* , 1992). These two categories of antigens are therefore of interest for future vaccine and epidemiological studies. Williamson *et al* (1989) described a MAb specific to *T.annulata* sporozoite, that neutralises their activity *in vitro*. The gene coding for the antigen recognised by the MAb was cloned, and a fusion protein expressed which elicited strong neutralising antibodies. Common and stage specific molecules identified by MAbs could be of potential use in diagnostic test, such as the Enzyme Linked Immuno Sorbent Assay (ELISA), to characterise the level of infection in endemic areas.

The characterisation of proteins recognised by the MAbs described in this study, presented difficulties. The two sets of proteins detected by MAbs 3D10 and 19E11 and MAbs 12D5 and 17H2, were difficult to transfer by blotting, partly due to their large molecular mass. The bands representing the polypeptides were often distorted (Fig. 3.2, track 5; Fig. 3.6, track 2) or not very well separated. Two major bands have been detected in *TaTu 2* protein extracts using MAbs 12D5 and 17H2 (Fig. 3.2, track 5), which were not separated in other blots as illustrated by Fig. 3.6 (Track 8). This was not due to the acrylamide concentration of the gel as a lower gel concentration also showed similar results (Compare Fig. 3.2, 10% gel with Fig. 3.6, 8% gel). This was not due to the sample concentration either, as lower concentrations of the initial samples (1/10) were used and no better separation or transfer of the proteins could be obtained (Fig. 3.7). These problems were considered when deciding what reagents would be used to analyse the stocks collected from Tunisia.

In conclusion, ten MAb raised against a *T.annulata*-infected cell line from Tunisia, were selected on the basis of their reactivity with the intracellular schizont. Six of them detected antigenic variants within the *T.annulata* stocks tested. The proteins recognised by six of the ten MAb were determined by Western blotting. The results showed that mixed infections with at least two antigenic variants were detected by these MAb.

The analysis of antigenic diversity has been shown to be important for epidemiological studies as well as strain characterisation of protozoan parasites. The importance of such analyses was also of interest in defining the extent of variability in polypeptide components of eventual subunit vaccines.

Given that this study showed that variability occurred within *T.annulata* isolates from one region, it was important to investigate the extent of diversity existing within a country using other markers. Other studies have illustrated the importance of isoenzymes in strain characterisation of other protozoan parasites (Sanderson *et al*, 1981; Babiker, *et al*, 1991b). Isoenzyme analysis has also been shown to be a practical assay to distinguish *T.annulata* stocks (Melrose, 1983). The use of isoenzyme electrophoresis to detect further variants of the parasite is described in the next chapter. The study of the diversity on a genomic level using DNA probes is also reported later in this thesis.

## ***CHAPTER FOUR***

### ***BIOCHEMICAL POLYMORPHISM***

## 4.1 INTRODUCTION

It is possible to assess the relatedness of organisms by comparing the structure of their homologous proteins, since these are modified by genetic procedures (Gibson *et al*, 1980). Enzymes are proteins easily identified by their substrate specificity. When the molecular structures of the enzyme are associated with a different charge, they can be separated by electrophoresis (in an electric field). If the supporting matrix used is a molecular-sieving medium, such as starch-gel, separation by size also occurs.

Enzymes have been widely used in biochemical characterisation of parasitic protozoa, particularly disease-causing agents in man. They have found many applications as characteristic diagnostic markers and some of them have elicited a degree of biological polymorphism of considerable interest in parasite differentiation. Trypanosomes (Godfrey, 1979), *Leishmania* spp (Chance *et al*, 1978), *Plasmodium* spp (Carter and Walliker, 1977) and *Eimeria* spp (Shirley, 1975) have perhaps been most extensively studied, but enzyme electrophoresis has more recently also been conducted in *Babesia* spp (Momen, 1979) and *Theileria* spp (Musisi, 1978).

Enzyme polymorphism, as revealed by electrophoresis, has yielded valuable information on many species of parasitic protozoa. Godfrey and Kilgour (1976) separated *Trypanosoma brucei gambiense* from *T.b.brucei* and *T.b.rhodesiense* by the characteristics of their aminotransferase patterns. Sargeaunt *et al* (1978) differentiated the invasive form of *Entamoeba histolytica*, causing clinical amebiasis, from the non-invasive form by electrophoresis of two enzymes. Al-Taqi and Evans (1978) identified *Leishmania tropica major* (rural) and *L.t.minor* (urban) by comparing their enzyme patterns with those of known stocks. Enzyme polymorphism has been of great value in epidemiological studies of many parasitic protozoa, e.g. *Plasmodium falciparum* (Carter and Voller, 1975; Sanderson *et al*, 1981, Babiker *et al*, 1991b), *Trypanozoon* (Gibson and Gashumba, 1983, Boid, 1988; Godfrey, 1990). The method allows many samples and often many characters to be compared quickly and easily. It also enables a direct assessment of the host cell and parasite enzyme contributions in case of intracellular parasites.

Very few studies have been carried out on isoenzymes in *Theileria* spp. Musisi *et al*



(1981) differentiated between *T.parva*, *T.lawrencei* and *T.annulata* by using three enzymes: glucose phosphate isomerase (GPI), glyceraldehyde phosphate dehydrogenase (GALDH) and aldolase (ALD). Only GPI showed some variation between *T.parva* stocks.

In a further study of 13 enzymes by isoelectric focusing (IEF), Van der Meer *et al* (1981) found that only GPI exhibited variation between *T.parva* stocks. This technique possesses a greater resolving power than simple electrophoresis but the patterns are complicated and more difficult to interpret.

A study on *T.annulata* (Melrose, 1983) showed that electrophoresis is a suitable method for the examination of parasite associated enzyme polymorphism. The author examined 22 enzymes but only GPI proved to be easily detected in all three stages of the parasite's life cycle: the intraileucocytic schizont, the intraerythrocytic piroplasm and the sporozoite in the vector tick. This enzyme also showed polymorphism between the 11 *T.annulata* stocks studied. Because of the variation seen in the GPI isoenzyme patterns, this enzyme is not suitable as a species marker (Melrose, 1983). However, in the same study, Melrose (1983) has shown that the lactate dehydrogenase (LDH) enzyme pattern is conserved between *T.annulata* stocks but different from the pattern exhibited by a single *T.parva* stock, making this enzyme a reliable marker for differentiating the two *Theileria* species.

Eighteen stocks of *T.annulata*, collected from five different countries, have been studied biochemically for GPI isoenzyme polymorphism using schizont-infected cells, piroplasms and sporozoite lysate (Melrose, CTVM, personal communication). These studies confirmed the ability of GPI analysis to detect polymorphism in *T.annulata* (Melrose, 1983).

In the present study, and given what has already been found, GPI enzyme electrophoresis was selected to analyse the large number of *T.annulata* stocks collected from Tunisia. The study was intended to identify the number of GPI variants circulating in a single country, to examine the relationship between these variants and specific geographical locations and finally, to analyse the extent of variation within a single isolate of *T.annulata*. The analysis concerned schizont-infected cell lines, piroplasms and sporozoites-infected tick salivary glands.

## **4.2 MATERIALS AND METHODS**

### **4.2.1 *Preparation of lysates for electrophoresis***

#### **4.2.1.1 Schizont-infected cells**

Schizont-infected cells, growing in culture, were washed twice in PBS, the pellet resuspended in 1ml of PBS and transferred to an Eppendorf tube. The cells were centrifuged at 1500 x g for 5 min and the supernatant discarded, with the last drop of fluid carefully removed. An equal volume of 5% triton x 100 in distilled water (Momen *et al*, 1975) was then mixed with the pellet. The suspension was subjected to three cycles of freeze-thawing at -70°C and centrifuged at 14000 x g for 5 min. The supernatant was then separated into 10ul aliquots and stored at -70°C to avoid repeated freeze-thawing of the sample.

#### **4.2.1.2 Piroplasms**

Lysates were prepared from purified piroplasms (Chapter 2, Section 2.2.6) by mixing with an equal volume of 5% Triton x-100. The suspension was treated as previously described for the schizont stage.

#### **4.2.1.3 Tick salivary glands**

Tick salivary glands were dissected out in PBS (Chapter 2, Section 2.2.4.1), blotted on the edge of the petri dish used for embedding the ticks, then dropped into an Eppendorf tube containing 5% Triton x-100. One to four pairs of salivary glands were resuspended in 10-30ul of 5% Triton x-100. The volume used was dependant upon the level of infection detected in the salivary glands. The suspension obtained was then treated as described in Section 4.2.1.1.

### **4.2.2 *Electrophoresis***

The method used for electrophoresis and specific enzyme staining in thin layer starch gel, has been described by Melrose and Brown (1979). The samples were separated by horizontal electrophoresis on thin layer starch gels (approximately 1mm thick) on glass plates.

Using the electrophoretic conditions described the parasite GPI is known to migrate towards the anode, while the host cell GPI migrates towards the cathode. The lysates were applied to the gel on white cotton threads and placed in slots cut 7.5cm from the cathodal end of the gel plate. The electrophoresis was performed at 8°C at 350V, for 3 hr with an initial current of 20mA per gel. The GPI activity was revealed using a developer containing 1ml methyl thiazolyl tetrazolium (4mg/ml), 30ul glucose-6-phosphate dehydrogenase (265u/ml), 1ml 0.1M MgCl<sub>2</sub>, 0.5ml NADP (6mg/ml), 0.5ml fructose-6-phosphate (5mg/ml), 4ml 0.3M Tris-HCl pH 8, 5ml H<sub>2</sub>O and 0.1ml meldola blue (6mg/ml) (Melrose *et al*, 1980). The developer was then mixed with 10ml of 1% agar (50°C) and poured on the top of the gel. The plate was incubated at 37°C until the bands of enzyme activity were visualised (30-45 min).

### 4.3 RESULTS

The Tunisian *T.annulata* stocks described in Chapter 6, have been analysed for the presence of GPI isoenzyme polymorphism. A number of different profiles and variants have been detected. They are illustrated in this chapter. The results showed that a minimum of a triplet of bands to a maximum of 9-10 bands, were detected in the stocks analysed. The profiles seen varied in both number and position of the bands revealed. Continuous profiles were composed of 3, 5-6, 7-8 or 9-10 successive bands. They comprised the majority of the stocks studied. Other profiles presented gaps (-) in between two sets of bands (Fig. 4.1).

As seen in Fig. 4.1, the bands detected also varied in intensity within and between the stock studied. Cloned macroschizont-infected cell lines invariably presented a three band profile, the position of the triplet varying in its electrophoretic migration between the different clones. Fig. 4.2 shows cloned infected cell lines derived from stock *TaTu* 11. The position of the basic three bands was also variable in the Tunisian stocks. Seven electrophoretically distinct forms of the triplet were detected (Fig. 4.3).

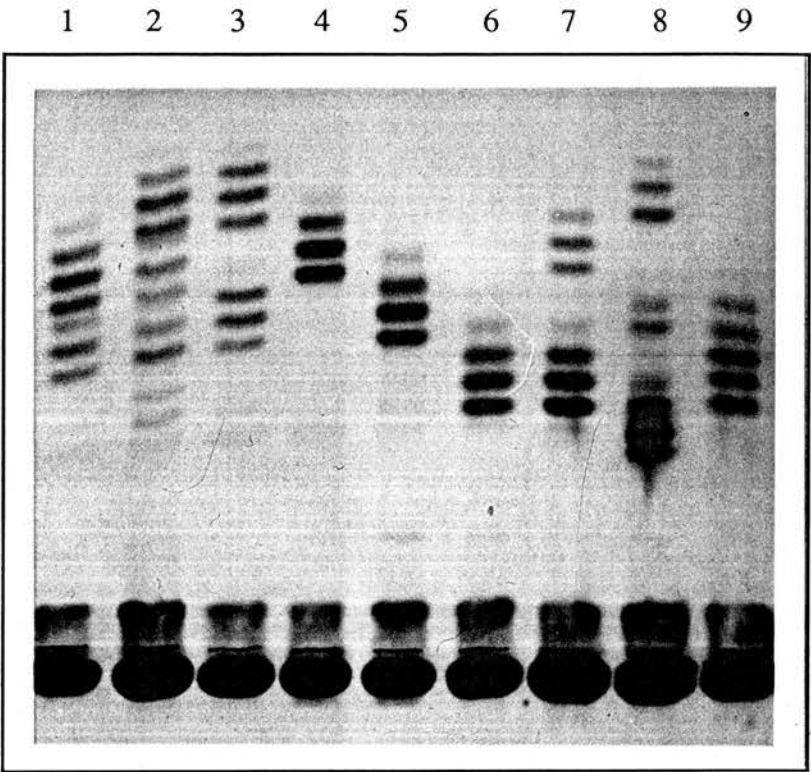
### 4.4 DISCUSSION

Previous studies on *Plasmodium* (Carter, 1970; Carter and Voller, 1975) and *Trypanosomes* (Gibson and Gashumba, 1983; Gibson and Wellde, 1985) showed variability

**Figure 4:1** Illustration of representative GPI enzyme patterns detected in the Tunisian stocks

Fig. 4.1	Track Number	<i>T.annulata</i> Infected Cell Lines	Patterns & Numbers of Bands Detected
A	1	<i>TaTu</i> 50	6
	2	<i>TaTu</i> 11	10
	3	<i>TaTu</i> 37	3-3
	4	<i>TaTu</i> 26	3
	5	<i>TaTu</i> 22	3
	6	<i>TaTu</i> 29	3
	7	<i>TaTu</i> 27	3-3
	8	<i>TaTu</i> 40	3 - 2 - 3
	9	<i>TaTu</i> 15	5
B	1	<i>TaTu</i> 40	3 - 2 - 3
	2	<i>TaTu</i> 11	10
	3	<i>TaTu</i> 18C	9
	4	<i>TaTu</i> 33	9
	5	<i>TaTu</i> 36	5 - 3
	6	<i>TaTu</i> 40	3 - 2 - 3

(A)



(B)

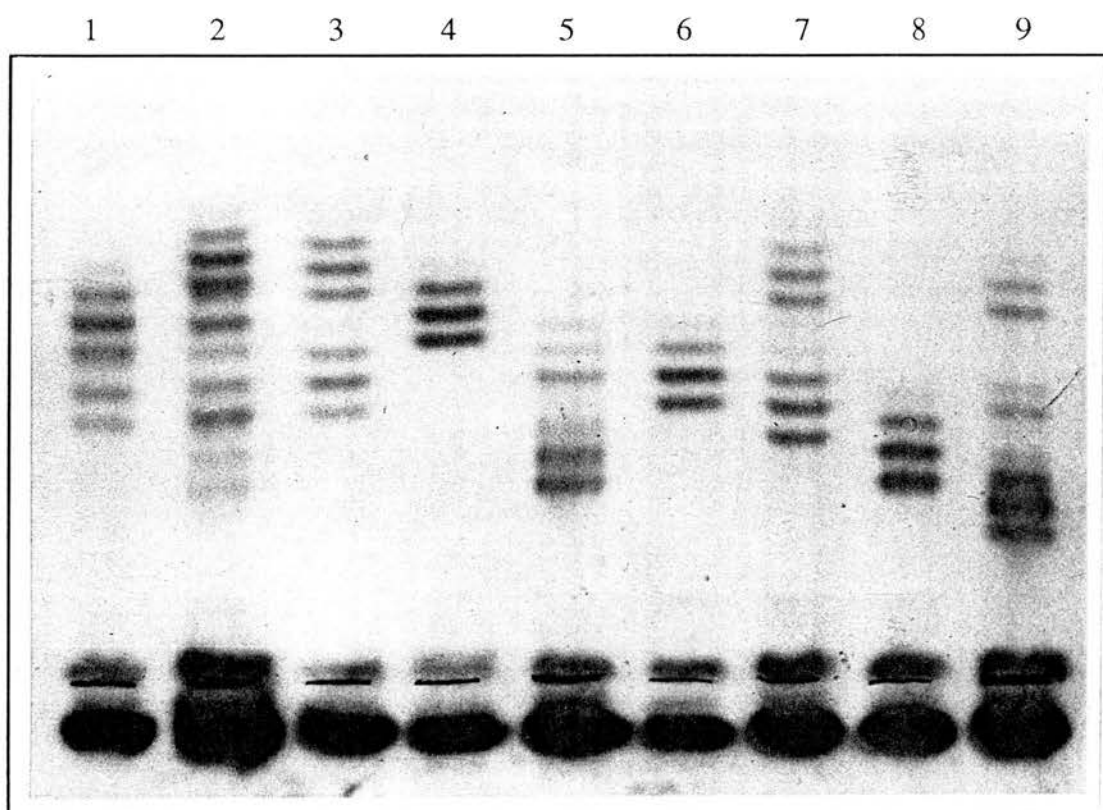
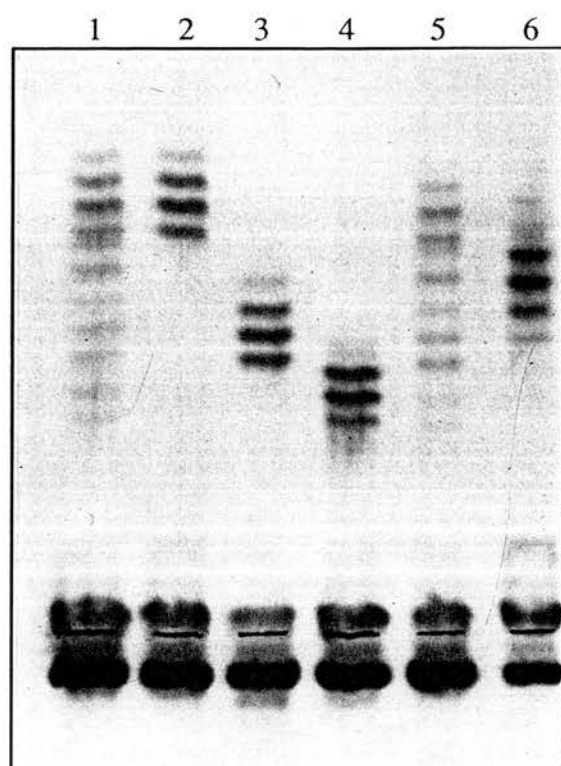


**Figure 4.2** GPI isoenzyme patterns of *T.annulata*. Tracks correspond to stocks.

Tracks	Stocks
1 and 5	<i>Ta</i> Tu 11 cell lines
2 - 4	<i>Ta</i> Tu 11 cell line clones
6	<i>Ta</i> Tu 11 piroplasms

**Figure 4.3** GPI isoenzyme patterns of *T.annulata*-infected cell lines illustrating the several variants detected amongst the Tunisian stocks, from the most anodal (1) to the most cathodal (7). Tracks correspond to stocks.

Tracks	<i>T.annulata</i> -infected cell lines
1	<i>Ta</i> Tu 50 (2 + 4)
2	<i>Ta</i> Tu 11 (1 + 2 + 4 + 6)
3	<i>Ta</i> Tu 37 (1 + 4)
4	<i>Ta</i> Tu 26 (2)
5	<i>Ta</i> Tu 16 (3 + 6)
6	<i>Ta</i> Tu 22 (4)
7	<i>Ta</i> Tu 18A (1 + 5)
8	<i>Ta</i> Tu 29 (6)
9	<i>Ta</i> Tu 40 (1 + 4 + 7)





in the mobility of isoenzymes, including GPI, derived from the parasites. Two forms of GPI were detected in *Plasmodium falciparum* (Carter and McGregor, 1973). The differences in electrophoretic mobility of these enzymes have been shown to be precise and stable characteristics of the parasite and were used as markers in genetic studies (Babiker *et al*, 1991b). In a study on *P.berghei* Carter (1970) showed enzymic differences between and within subspecies using observations on the electrophoretic forms of four enzymes.

Enzyme variation in *Theileria* is insufficiently documented. Variant forms of GPI were reported for *T.parva* (Musisi 1978; Van der Meer *et al*, 1981). However this characteristic has been poorly used in strain characterisation. Differences between *T.parva* and *T.annulata* using GPI analysis have also been reported (Melrose and Brown 1979; Musisi *et al*, 1981). However the polymorphism in *T.annulata* makes it an unreliable enzyme to choose to distinguish between the two species. A total of 18 stocks of *T.annulata* from five countries were analysed prior to this study (Melrose, unpublished data), they were shown to be highly heterogeneous using GPI isoenzyme studies. Over one hundred cloned cell lines deriving from these stocks were also examined and three different electrophoretic forms of the GPI isoenzyme were detected (Wilkie *et al*, 1986). These were represented by a basic triplet pattern.

Given these studies, GPI isoenzyme was chosen to detect *T.annulata* variants in Tunisian stocks. The patterns were difficult to analyse and in an attempt at simplifying the results, a system was used whereby the variant forms of the triplets detected were numbered from the most anodal (1) to the most cathodal (7). These numbers were then used to characterise the stocks. For example stock *TaTu* 11 (Fig. 4.1B, Track 2) was referred to as, 1+2+4+6.

Seven variants (of the electrophoretic form of the GPI enzyme) were detected in the stocks analysed (Fig. 4.3), and a maximum of 10 bands were identified in a single stock. The most satisfying hypothesis to explain these profiles was that the bands seen are the result of overlapping bands of different variants, with each variant possessing a basic triplet pattern as shown in the following diagram:

Variant Number	1	2	3	4	5	6	7
-							
-							
-		-					
		-	-				
		-	-	-			
			-	-	-		
				-	-	-	
					-	-	
						-	-
							-
							-

Hence, some of the bands seen would represent two proteins each from a different variant but migrating at the same point. This is supported by the fact that sometimes a doublet of very close proteins was detected, indicating the presence of two distinct triplets possessing successive electrophoretic migration (Fig. 4.1A, Track 1). In contrast, when the triplet were overlapping, only five bands could be detected (Fig. 4.1A, Track 9). The lack of sharpness of some bands, most probably arising from the close migration of two bands, would support this explanation (Fig. 4.3, Track 4).

This hypothesis was not entirely satisfactory. the variation in intensity of certain bands within a stock and particularly within a triplet (Fig. 4.1B, Tracks 5 and 6) was sometimes apparent as well as the detection of only two bands within some variants (Fig. 4.1B, Track 6). However, the bands detected in stocks composed of a single triplet or in cloned cell lines, were always of equal intensity and invariable number (3 bands). This would indicate that the difficulty in detecting one band in some triplets could be due to there being an insufficient amount of enzyme for detection in the experimental conditions prevailing. Indeed Godfrey (1990) reported that the low concentration of enzyme in the extract may affect the resolution of certain bands, which may not be detected.

The presence a very faint band at the top of the pattern presented by certain stocks (Fig. 4.1A, Tracks 5 and 6) was probably due to the extraction procedure needed to bring the

enzymes into solution, or to the storage conditions, which may have resulted in partial degradation as explained by Moss (1979). Many comparative electrophoretic analyses of the same stock were often required to detect these artefactual modifications.

From the cloning experiments conducted in this study (Fig. 4.2) and previous studies (Wilkie, 1986; Shiels *et al*, 1992) it has been shown that a cloned infected cell line consistently presents a three band pattern. Most authors are of the opinion that the bovine stages of *T.annulata* are haploid (Morzaria *et al*, 1992a) although proof is still lacking. In this condition each parasite clone is represented by a single form of the enzyme. The seven different electrophoretic forms of the GPI enzyme detected in *T.annulata* in this study, would then correspond to seven allelic variants. If the GPI complete enzyme molecule consists of two polypeptides subunits (AB), the dimeric enzyme could be formed by the mixed polypeptides (AB), added to the dimers, composed of pairs of identical subunits (AA and BB). Thus three different molecule forms could be expected for a GPI enzyme. These would correspond to the three bands detected by electrophoresis. The middle band representing the mixed dimer.

The detection of all the variants present within one enzyme pattern was not accurate, since it was only based on the number of bands detected. GPI variants 3 and 5 would not be detected if mixed with, respectively, variant 2, 4 and 4, 5 (Fig. 4.3). Therefore the characterisation of stocks composed of more than one population, using the number of bands visualised, might not reflect the real number of variants present within these stocks. This would particularly hold for new, undetected variants. This method was, however, used because of its simplicity, to describe the stocks studied. The frequency with which particular variants occurred within the stocks, has been analysed and was reported in Chapter 6.

As the results showed a maximum of 9-10 bands within one isoenzyme pattern, this would fit with the detection of at least four different variants. Since each variant corresponds to one parasite population, as demonstrated by cloning, a mixture of at least four different subpopulations of *T.annulata* parasite were detected in single representatives of the Tunisian stocks. Other associations of two or three variants were also present, together with the individual profile pattern. In these individual patterns the absolute evidence of the presence

of only one population has to be confirmed by other markers as discussed in Chapter 6.

Overall this study of GPI electrophoretic patterns within the Tunisian *T.annulata* stocks has shown new evidence for:

- (i) The existence of at least seven different parasite subpopulations (Fig. 4.3).
- (ii) Under field conditions, a single animal may be simultaneously infected with at least four of these variants (Fig. 4.1B).

A genetic cause of enzyme variation can often be concluded from studies of the multiple forms detected in a population (Carter and McGregor, 1973; Babiker *et al*, 1991b), but the definitive evidence for the genetic origin of the enzyme variation would be in demonstrating the differences existing in the basic structure of the enzymes. However, todate, this has only been achieved in a few cases (Moss, 1979).

*T.annulata* diversity has been investigated on the immunological level and the biochemical level. This investigation would not have been complete without taking it further and evaluating the variability at the genomic level. Therefore, in the next chapter, analysis of the polymorphism of, and diversity within, *T.annulata* using genomic probes is described.

## ***CHAPTER FIVE***

### ***ISOLATION AND CHARACTERISATION OF DNA PROBES***

## 5.1 INTRODUCTION

The aim of this study was to develop a system for comparing *T.annulata* stocks at the genomic level, after analysing the variation at the immunological and biochemical levels. Previous studies of parasitic protozoa have shown that of the three approaches cited above, methods of stock characterisation by direct analysis of the parasite genome are able to detect a greater level of polymorphism. Most of these studies have been based on variation in repetitive DNA sequences and the analysis of DNA restriction fragment length polymorphisms (RFLP). Such studies, on a range of parasitic protozoa have been shown to be a valuable means of distinguishing species and strains. The most frequent approach to examine levels of polymorphism was the detection of restriction endonuclease variation by hybridisation with DNA probes. RFLP and hybridisation has been used to differentiate between species, strains and eventually clones of *Plasmodia* (McCutchan *et al*, 1984; Coppel *et al*, 1985; Hommel *et al*, 1991; Ranford-Cartwright *et al*, 1991; Babiker *et al*, 1991a), *Trypanosoma* (Paindavoine *et al*, 1986, Hide *et al*, 1991; McDaniel and Dvorak, 1993), *Leishmania* (Jackson *et al*, 1986; Van Eys *et al*, 1991) and *Babesia* (Posnett and Ambrosio, 1989; Dalrymple, 1990).

The first study on *Theileria* was performed by Conrad *et al* (1987a) who showed polymorphism between five different stocks of *T.parva* using RFLP and hybridisation to DNA either from intraerythrocytic piroplasms or macroschizont-infected cell lines with two repetitive DNA probes. DNA probes were also used in studies aimed at discriminating parasites of the *T.parva* complex which has been divided into three subspecies on the basis of the clinical and epidemiological characteristics of the parasite in cattle (Uilenberg, 1981). They did not show any specific characteristic that would distinguish the three subspecies genotypically (Conrad *et al*, 1989, Allsopp *et al*, 1989). However these methods have been useful in detecting the carrier state in *T.parva* infected cattle using the polymerase chain reaction (Bishop *et al*, 1992) and in the identification of four chromosomes in *T.parva* (Morzaria *et al*, 1992a). DNA probes were also used to detect *T.parva* in tick salivary glands (Chen *et al*, 1991). Genomic analysis of *T.sergenti* stocks with DNA probes showed polymorphism between field isolates (Matsuba *et al*, 1992) and changes in the hybridisation patterns of the parasite in individual animals (Matsuba *et al*, 1993).

Few studies have been carried out on *T.annulata* genomic diversity. Williamson *et al* (1989) showed differences between two stocks of *T.annulata* using a sporozoite antigen gene probe (SPAG-1). The presence of mixed parasite populations in *T.annulata* using DNA probes has been shown by Shiels *et al* (1992) within *T.annulata* Ankara cloned infected cell lines using one probe raised in this study (TaT 16).

The detection of polymorphism in *T.annulata* between more than two stocks using DNA probes has not been described before. This work accordingly aimed to investigate the variation in *T.annulata* parasites using a large number of stocks from a single country. The following questions were examined:

- (i) What is the level of diversity showed by the parasite?
  - within a country
  - within a bioclimatic zone
  - within a district
  - within a farm
- (ii) How many genotypes are circulating within these different habitats?
- (iii) How many of genotypes are found within a single parasite isolate?

This chapter describes the preparation and selection, using an initial study on a few isolates, of the DNA probes used to ask these questions. It also reports the DNA extraction and the hybridisation procedures.

## **5.2 MATERIALS AND METHODS**

### **5.2.1 Parasite Material**

The Tunisian stocks described in this chapter are listed in Table 5.1. They consist of five macroschizont-infected cell lines and the piroplasms isolated from the same animals, one piroplasm preparation (TaTu 7) used to construct a *T.annulata* genomic library and a macroschizont-infected cell line (TaTu 2). All these preparations were isolated from animals



**Table 5.1**     *T.annulata* stocks used to characterise the DNA probes.

<i>TaTu</i> stocks	Origin of DNA extract		Comments
	Cell Line	Piroplasms	
1/270	+	+	Experimental isolate
2	+	-	Field isolate
2/029	+	+	Experimental isolate
7	-	+	Field isolate
9A }	+	+	Field isolates
9B }			
10 }			
13 }			

undergoing clinical theileriosis either naturally (field isolate) or after experimental infection with a macroschizont-infected cell line (experimental isolate).

Laboratory maintained stocks from different countries and clones derived from them as macroschizont-infected cell lines were also used in this chapter to define the reactivity of DNA probes. These were *T.annulata* Ankara from Turkey (Schein, 1975) and derived clones C9, E3, D3 and D7 (Shiels *et al*, 1992), *T.annulata* Hissar from India (Gill *et al*, 1976) and derived clones A1, A6, B1 and A12 (Wilkie, unpublished data), *T.annulata* Gharb from Morocco (Ouhelli, 1985) and *T.parva* Mugugua from Kenya.

### 5.2.2 DNA extraction

DNA was obtained from three parasite stages as described in Chapter two: sporozoites (infected tick salivary glands, Section 2.2.4), macroschizont-infected cell lines (Section 2.2.5) and piroplasms (Section 2.2.6). The two bovine stages were extensively studied especially the intracellular macroschizont which was the main source of parasite material from the field isolates.

The DNA was extracted according to standard procedures (Maniatis *et al*, 1982; Conrad *et al*, 1987a; Van Eys *et al*, 1989). Piroplasms, schizont-infected cells or PBM were suspended in lysis buffer (50mM NaCl, 10mM EDTA pH 8, 50mM Tris-HCl pH 7.6). Then 0.5% SDS in distilled water was added immediately and the suspension mixed gently. Proteinase K (Sigma) was added to a concentration of 100ug/ml and the preparation incubated for 2-3h at 55°C then overnight at 37°C. The DNA was extracted once with phenol and once with phenol/chloroform (1:1). At this stage RNase A (Boehringer Mannheim) (boiled in 10mM Tris Hcl pH 7.5, 15mM NaCl for 15 min) was added to a concentration of 20ug/ml and the mixture incubated for 1h at 37°C. The DNA solution was then extracted with chloroform/isoamyl alcohol (v/v, 24:1) and precipitated, after addition of sodium acetate to a final concentration of 0.3M, by two volumes of ice cold ethanol. The DNA was left overnight at 4°C in ethanol, centrifuged (2000 x g, 15 min), the pellet rinsed with 70% ethanol and centrifuged using the same conditions, dried for 1-2hr at room temperature and dissolved in 10mM Tris HCl pH 7.6, 1mM EDTA pH 8 (T.E.). The concentration was measured, after the

DNA dissolved (1-2 weeks at 4°C), by its absorbance (O.D.) at 280nm and the purity by the ratio OD260/OD280. All glassware, plasticware and solutions used were sterile and handled with gloves.

The DNA was incubated with restriction enzymes *EcoRI*, *Hinf I* and *Hind III* (BRL) as per manufacturer's recommendations. Ten to 20ug of schizont-infected cell lines or PBM, 2-5ug of piroplasm or sporozoite DNA were digested with restriction enzymes overnight at 37°C and the samples were mixed with gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficoll in water).

### **5.2.3 Gel electrophoresis and Southern blotting**

The DNA was subjected to electrophoresis at 40mA in a 0.9% agarose gel prepared in Tris-borate (TBE: 0.045M Tris-borate, 0.001M EDTA) containing 1ug/ml of ethidium bromide. Electrophoresis was performed in submarine gels in 1 x TBE.

Prior to the transfer, and to improve its efficiency the gels were treated with acid (depurination) and base (hydrolysis) solutions. This depurination/hydrolysis treatment reduced the problem of dehydration of the gels during the transfer. The agarose gels were depurinated using a 0.25N HCl solution (2 x 15min), rinsed with distilled water, denatured using a 0.5M NaOH, 1M NaCl solution (2 x 15min), rinsed with distilled water, then neutralised for 1h with 3M NaCl, 0.5M Tris pH 7.4 solution. The transfer of DNA to solid supports (Southern, 1975) was carried out overnight using nylon membrane (Hybond, Amersham) in 20 x SSC buffer (1 x SSC = 0.15M NaCl, 0.015M sodium citrate pH 7.0). After the DNA has been transferred, it was fixed by exposure of the nylon filters, wrapped in saran wrap, to ultraviolet irradiation for 5 min. The filters were either hybridised or kept at -20°C until needed.

### **5.2.4 The hybridisation procedure.**

The filters were prehybridised in hybridisation mix (0.5M Na<sub>2</sub> HP04 pH 7.0, 7% SDS, 2mM EDTA) (Church and Gilbert, 1984) for 1hr, then the solution changed and the labelled probe added. The hybridisation was carried out overnight at 68°C.

The filters were washed for 4 x 15min at 68°C. The two first washes were carried out in 40mM Na<sub>2</sub> HPO<sub>4</sub> (pH 7.0), 2mM EDTA, 5% SDS, followed by two washes in 1% SDS. The filters were then briefly dried on filter papers and placed damp in saran wrap. They were exposed to X-ray film to obtain autoradiograms. The exposure time varied from a few hours (piroplasms) to three days (macroschizont-infected cell lines) at -70°C.

#### **5.2.5 Preparation of DNA probes.**

##### **5.2.5.1 Construction and screening of a *T.annulata* genomic library.**

DNA probes were generated from a genomic library constructed in *E.coli* and using a plasmid vector.

#### **a) Bacterial strain**

The *E.coli* strain used, TG1, is a derivative of strain JM101 that neither modifies nor restricts introduced DNA. The bacteria were grown in Lauria Broth (LB) medium and plated on LB agar (both from Difco).

#### **b) Plasmid vector**

Two vectors have been used in this study, pUC 18 and pBluescript 11 SK. Both plasmids replicate at a high copy number as they lack the gene involved in the control of copy number. pUC 18 has a poly cloning site containing, in particular one *Eco*RI site and one *Hind* III site. This plasmid was used to clone *Eco*RI digests of *T.annulata* DNA. The pBluescript 11 SK, derived from pUC 19, was used to subclone DNA probes for sequencing analysing. Both vectors contain an ampicillin resistance gene for antibiotic selection of the transformed bacteria.

#### **c) Construction and screening of the library**

Piroplasms purified from stock *Ta*Tu 7 were used to prepare genomic DNA. This DNA was digested and fragments inserted into the poly cloning site of pUC 18 by ligation. Both plasmid and genomic DNA were digested with *Eco*RI which resulted in the production of identical protruding termini. Fifteen ul (20µg) of DNA from the genomic and plasmid digests were mixed, then 1ul (0.5-2U) of bacteriophage T4 ligase (BRL) and 4ul of 5 x ligase

buffer (BRL) were added to make up a final volume of 20ul. The mixture was incubated at 12°C and added to TG1 competent cells prepared as follows.

The bacteria were grown in LB medium overnight, diluted (x10) and grown for another two to three hours at 37°C without exceeding  $10^8$  viable cells/ml. Fifty ml of bacterial suspension were cooled on ice for 10 min, centrifuged (2000 x g, 15 min), resuspended in 10ml of ice-cold 0.1M CaCl<sub>2</sub>, stored on ice for 10 min and centrifuged (2000 x g, 15 min). The resulting pellet was resuspended in 1ml of ice-cold 0.1M CaCl<sub>2</sub>. At this stage the cells were competent and ready for the transformation.

Approximately 50ng of ligated DNA, in a volume not exceeding 10ul, were added to 200ul of competent cells. The transformation was carried out using a positive control with a known amount of plasmid, without insert, at a ratio of 1ug of plasmid DNA per 50ul of competent cells. The suspensions were transferred at 42°C for 2 min, then rapidly cooled on ice for 1-2 min. The cells were then diluted in LB medium (1/5), incubated at 37°C for 30 min, plated (100ul/plate) on LB agar containing 100ug/ml ampicillin and incubated overnight at 37°C. Colonies of bacteria were picked at random and each colony grown in 5ml of LB medium with ampicillin, overnight at 37°C.

The bacterial suspensions were used to prepare small-scale preparations of plasmid DNA (see below) which were then digested with *EcoRI* and analysed by electrophoresis for the presence of inserts. Recombinant bacteria, containing inserts, were expanded on LB agar with ampicillin (100ug/ml), individual colonies grown in LB medium with ampicillin, and the bacterial suspensions were cryopreserved.

d) Storage of bacteria.

Bacteria were frozen in glycerol. One colony of bacteria was grown overnight in 5ml of LB medium with (for recombinant bacteria) or without ampicillin and 0.85ml of bacterial suspension were added to Eppendorfs containing 0.15ml of sterile glycerol. The suspension was mixed and stored at -70°C.

When needed the bacteria were recovered by scraping the surface without thawing the frozen culture, streaked on LB agar and incubated overnight at 37°C. The bacterial aliquot, kept frozen, would then be returned to -70°C for further use.

e) Extraction and purification of plasmid DNA.

The method used in this study was small-scale preparation of plasmid DNA. Minipreparations were obtained by the alkaline lysis method (Maniatis et al, 1982). Briefly, the recombinant bacteria were grown in LB medium with ampicillin overnight at 37°C and recovered by centrifugation (2000 x g, 15 min). The cells were lysed by detergent (1% SDS) and alkali (0.2N Na OH) treatments in presence of EDTA (10mM). The DNA was extracted by an equal volume of phenol/chloroform, the aqueous layer recovered, precipitated with two volumes of ethanol and centrifuged. The pellet was rinsed in 70% ethanol, centrifuged and dried in air for 10 min. The plasmid DNA was dissolved in T.E. (pH 8.0) containing RNase (20ug/ml) and stored at -20°C.

**5.2.5.2 Labelling of DNA probes.**

Minipreparations of plasmid DNA estimated to contain 20-30ug DNA per 100ul were used to prepare probes. Approximately 4ug of recombinant DNA were digested with 10U of *EcoRI* as per manufacturer's recommendations (BRL). The DNA probes were labelled by the random priming method using a commercial kit (Boehringer, Mannheim).

Twenty ul of digested recombinant plasmid were boiled, chilled on ice and 9ul of an equal mixture of the unlabelled oligonucleotides (A, G, T) was added together with 6ul of reaction buffer (10x) and 16ul of distilled water. Lastly 6ul (60uCi) of (alpha P<sup>32</sup>) dCTP and 3ul of Klenow enzyme were added, the solution mixed gently and incubated for 30 min at 37°C. The reaction was stopped by adding 20ul of 0.5M EDTA and 120ul H<sub>2</sub>O, to give a final volume of 200ul. This solution (the probe) was boiled for 5 min, chilled and added to the filters already incubated at 68°C in a fresh hybridisation solution (see section 5.2.4). The hybridisation was carried out and the filters washed as previously described in Section 5.2.4.

To estimate the specific activity of the labelled probe, one µl of the labelled mixture was dropped on a small piece of Whatman GF/C glass-fibre filter, dried and counted (hand-monitor). The filter was then soaked in ice-cold 10% TCA for 15 min, dried and counted again. On average the counts obtained from 1ul sample were 10<sup>6</sup>cpm.

### 5.2.6 *Additional probes used.*

Two DNA probes, SPAG-1, the sporozoite antigen gene probe (Williamson *et al*, 1989) and E2.1, a merozoite antigen gene probe of 1Kb (Dr B.R.Shiels, unpublished data), were also used. They were kindly provided by Drs Jane H. Kinnaird (Wellcome Unit of Molecular Parasitology) and Brian R. Shiels (Veterinary Parasitology), Glasgow University. The probes were labelled as described above and used for hybridisation to blots.

### 5.2.7 *Sequencing of DNA probes.*

Only partial sequencing of one of the probes used in this study was carried out. Suitable restriction fragments of probe *TaT* 17 were subcloned into a phagemid vector, pBluescript 11 SK with a multiple cloning site flanked by T3 and T7 promoters. Two fragments of, respectively, 0.7Kb (F1) and 1Kb (F2) were isolated from a 0.7% low melting agarose gel (LMA) used to run a double digestion of *TaT* 17 with *Eco*RI and *Hind* III restriction enzymes. F1 and F2 were purified from the agarose by the gene clean procedure as per manufacturer's recommendations (Gene clean, Stratech Scientific). The phagemid was digested with the two enzymes and run onto a LMA gel and isolated as described for F1 and F2. The fractions represented approximately 30% of the phagemid (2.961Kb). The ligation was carried out using a ratio of 1/1 between the vector and the insert (12ng/12ng). The amount of DNA in both inserts and the vector was estimated visually from the LMA gel by comparison to a known quality of control plasmid. Purified preparations of both fractions were incubated with pBluescript and T4 DNA ligase (0.5-2u) overnight at 12°C. Transformation was performed as described before and recombinant bacteria were used to prepare preparations of recombinant vector DNA. Templates for DNA sequencing consisted of double stranded DNA of F1 and F2 that had been denatured by 2M NaOH followed by neutralisation with 5M ammonium acetate pH 7.4. The DNA was precipitated at -70°C for 1h with absolute ethanol, pelleted and washed again with absolute ethanol, dried and resuspended in distilled water. The DNA was sequenced using the chain-termination method (Sanger *et al*, 1977) which has been fully described by Maniatis *et al*, (1982). The DNA sequencing was completed from each side of the inserts by using two synthetic oligonucleotides



complementary to T3 and T7 promoters, with sequenase version 2.0 (United States Biochemical).

### 5.2.8 Terminology.

The probes used in this study were named *T.annulata* Tunis (*TaT*) followed by the probe number. The numbers were assigned on the basis of the size of the insert from the smallest fragment 0.9Kb (*TaT* 1) to the largest one 8.2Kb (*TaT* 21).

## 5.3 RESULTS

Forty-eight colonies were analysed and from these 21 had plasmids containing visible inserts on agarose gels (data not shown). The insert length ranged between 0.9Kb-8.2Kb. Two colonies had plasmids with inserts that contained internal *EcoRI* sites, one with a single site and the second with two sites.

The aim of this study was to select a suitable probe for detecting polymorphism between and within the Tunisian stocks. Only four probes out of the 21, chosen at random, were examined for polymorphism by screening *EcoRI* and *Hinf I* digests of the DNA from the panel of stocks listed in Table 5.1, using Southern blotting and hybridisation with the labelled inserts of each. These were *TaT* 16 with an insert of 6.7Kb; *TaTu* 17, 6.8Kb; *TaTu* 20, 8Kb and *TaTu* 21, 8.2Kb. None of the inserts showed any hybridisation signal with uninfected bovine DNA (results only showed for probe *TaT* 16 in Fig. 5.2, track 8).

### 5.3.1 Probe *TaT* 16.

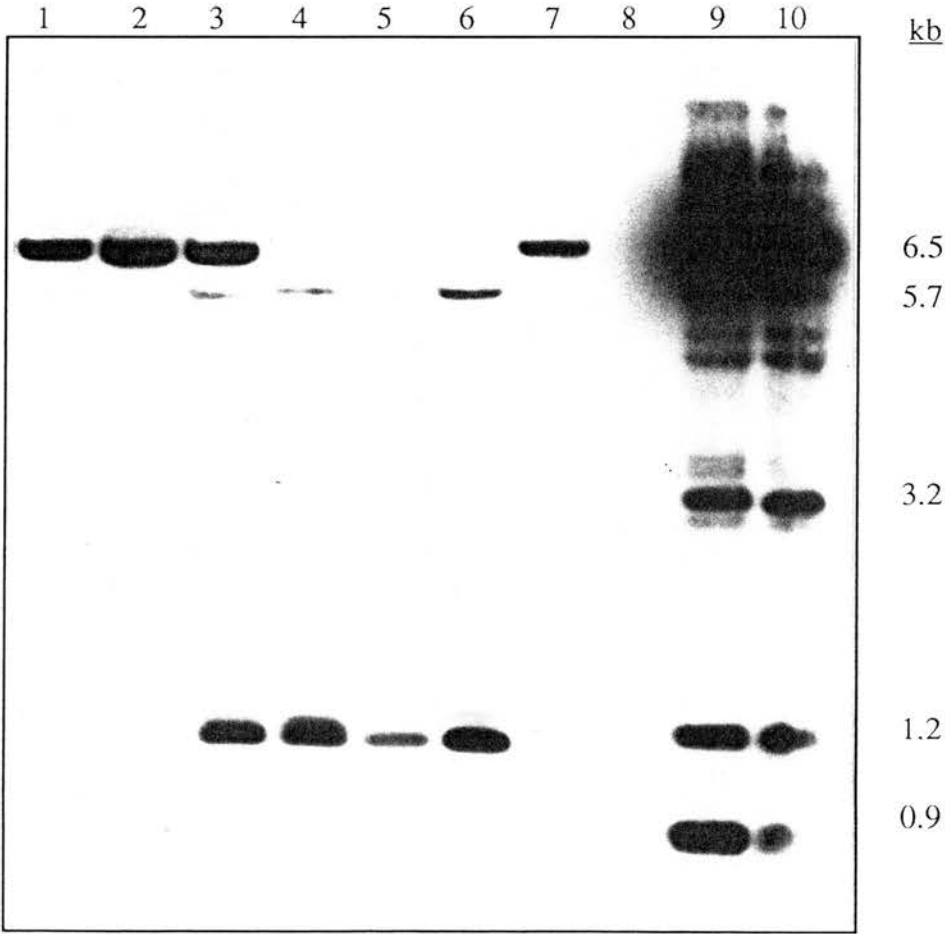
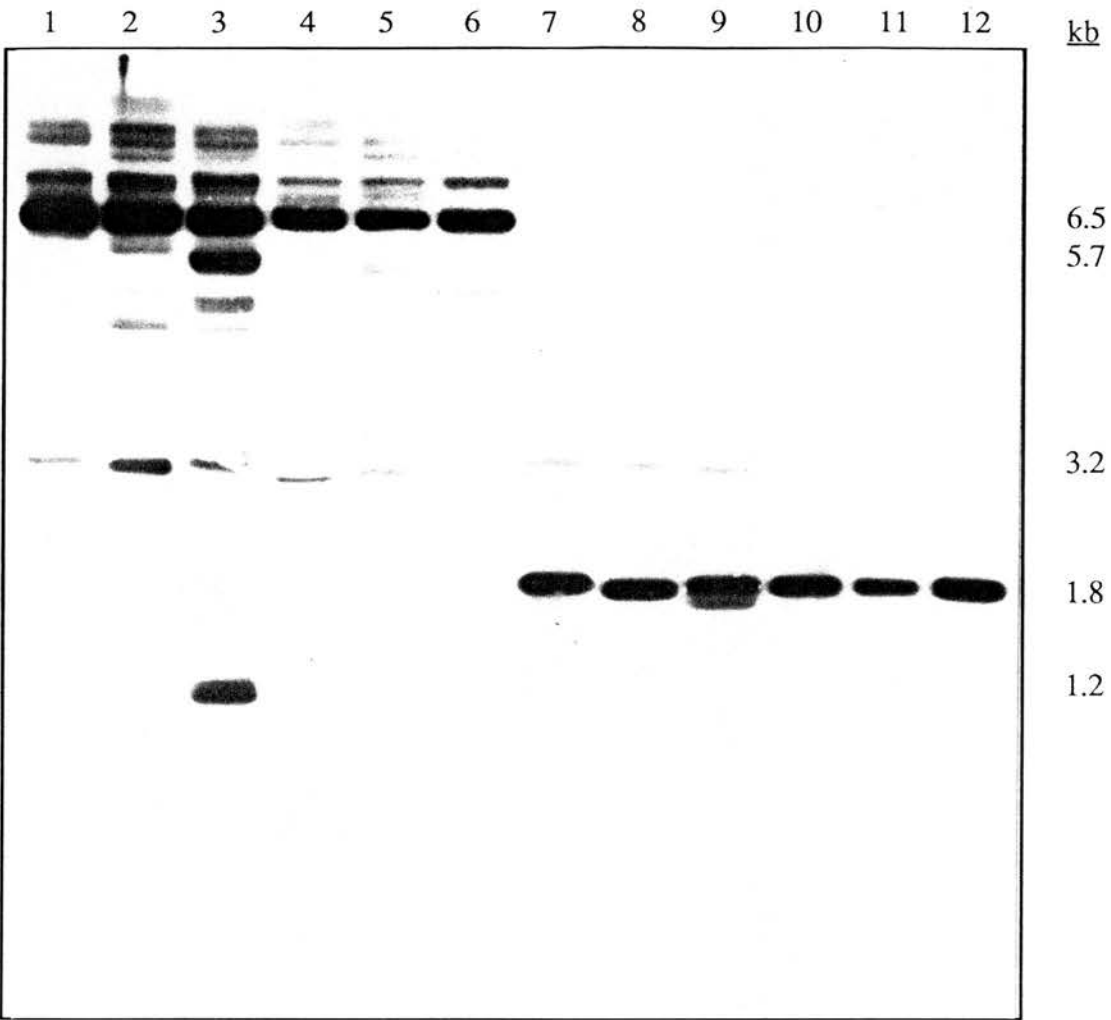
Fig. 5.1 shows the profile patterns obtained from piroplasm DNA digested with *EcoRI* (track 1-6) and *Hinf I* (track 7-12) hybridised with probe *TaT* 16. The *Hinf I* patterns of the different stocks were very similar, showing a single strongly hybridising fragment of 1.8Kb and a number of fainter fragments which varied in size between the stocks. Several fragments of variable intensity were detected on *EcoRI* digests of the same stocks. Four main fragments showed a strong hybridisation signal. The strongest fragment of 6.5Kb was present in all the stocks, whereas the 1.2Kb and 5.7Kb fragments were only present in stock *TaTu* 2/029 (track

**Figure 5.1** Southern blot of *T.annulata* piroplasms digested with *Eco*RI (tracks 1-6) and *Hinf* I (tracks 7-12), and hybridised with probe *TaT* 16. Tracks correspond to stocks.

Tracks	<i>T.annulata</i> piroplasms
1 and 7	<i>TaTu</i> 1/270
2 and 8	<i>TaTu</i> 7
3 and 9	<i>TaTu</i> 2/029
4 and 10	<i>TaTu</i> 9A
5 and 11	<i>TaTu</i> 9B
6 and 12	<i>TaTu</i> 10

**Figure 5.2** Southern blot of *T.annulata*-infected cell lines and piroplasms digested with *Eco*RI and hybridised with probe *TaT* 16. Tracks correspond to stocks.

Tracks	Stocks
1 and 2	<i>TaTu</i> 1/270 cloned cell lines
3	<i>TaTu</i> 2 cell line
4	<i>TaTu</i> 2 cloned cell line
5	<i>TaTu</i> 2/029 cell line
6	<i>TaTu</i> 2/029 cloned cell line
7	<i>TaTu</i> 1/270 cell line
8	Uninfected PBM
9 and 10	<i>TaTu</i> 1/270 piroplasms



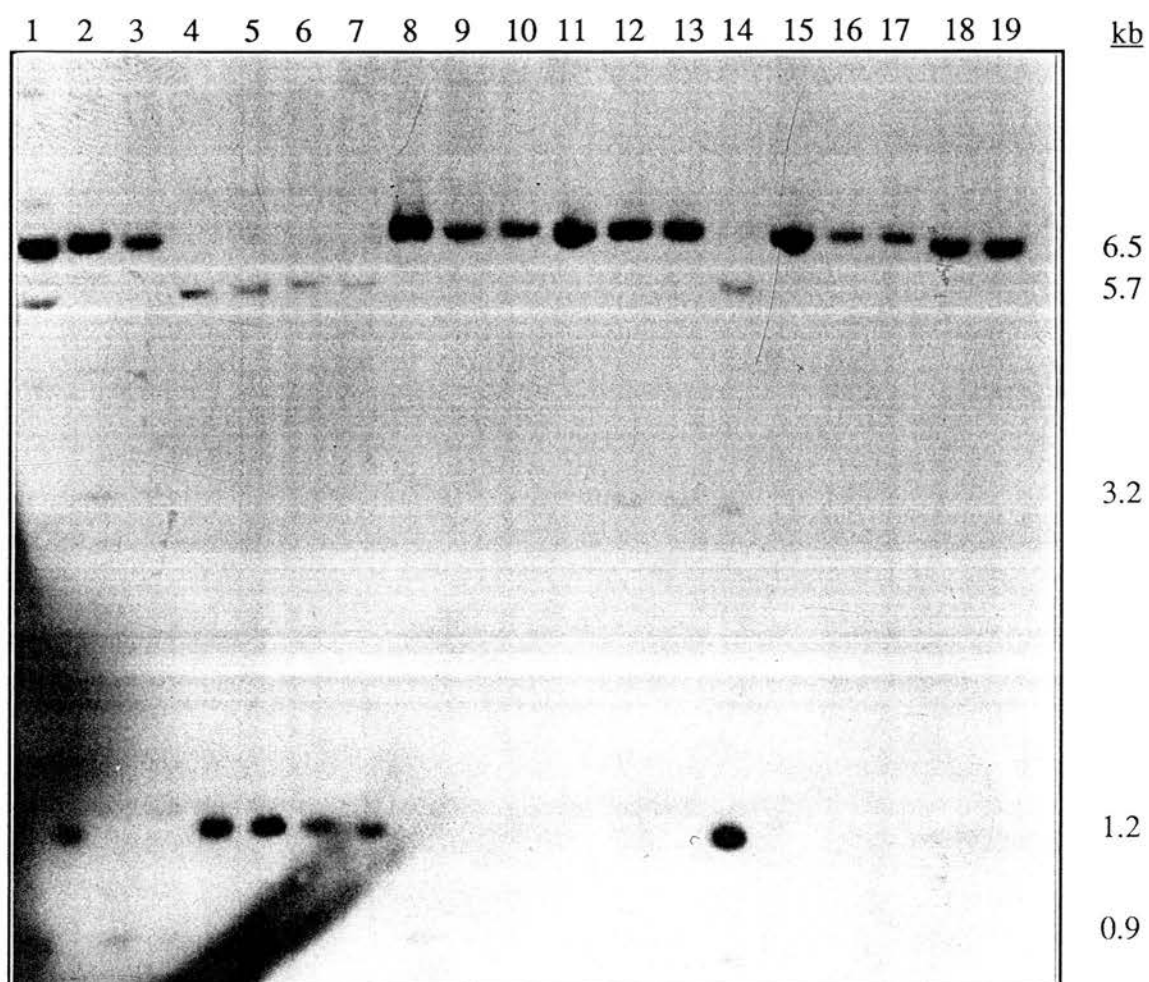
3). All stocks had the 3.2Kb fragment which showed a strong hybridisation signal in stock *TaTu* 7. The other fragments seen on *EcoRI* digests in Figure 5.1 showed variation in both their presence and the intensity of hybridisation signal given.

Fig. 5.2 compared patterns obtained with DNA from uncloned infected cell lines, cloned infected cell lines and piroplasms. Three fragments detected in the piroplasm DNA, were present in the cell line preparations. These were all detected in cell line *TaTu* 2 and are of 6.5Kb, 5.7Kb and 1.2Kb in size (track 3). Only the 5.7 and 1.2Kb fragments were present in *TaTu* 2 clone, (track 4) *TaTu* 2/029 (track 5) and its derived clone (track 6). The minor fragments seen in cell line DNA extracts were not noticed on other Southern blots (see Fig. 5.3). They were thought to represent non-specific background binding of bovine DNA, since, for example, the minor bands seen at approximately 1.2Kb, correspond to bovine satellite DNA on agarose gels. Cell line and piroplasm DNA from stock *TaTu* 270/1 presented different patterns. Only one of the fragments seen in the piroplasm DNA, (6.5Kb) was observed in the cell line. (Compare tracks 7 and 9 in Fig. 5.2). Comparison of the results with the piroplasm DNA from this stock in Fig. 5.1 (track 1) and Fig. 5.2 (tracks 9 and 10) shows two differences. Two additional fragments of 1.2Kb and 0.9Kb were detected in Fig. 5.2. Both preparations were made from the same DNA extract and digested with the same enzyme (*EcoRI*) under the same conditions.

The profile of fragments detected by *TaT* 16 was examined using stocks from different countries and their derived clones as illustrated in Fig. 5.3. Two strongly hybridising fragments of 6.5Kb and 1.2Kb were detected, together with a fragment of intermediate intensity at 5.7Kb and two very faint fragments of 3.2Kb and 0.9Kb (Fig. 5.3). In *T.annulata* Ankara (Fig. 5.3, track 1), three fragments of 6.5Kb, 5.7Kb and 1.2Kb were detectable. The clones derived from this stock show two different profiles. Clone C9 and D7 showed the 6.5Kb and the two faint fragments of 3.2Kb and 0.9Kb (Fig. 5.3, track 2. Bands in track 8 were faint and can not be seen in the figure), whereas clone E3 and D3 showed the 5.7Kb and 1.2kb fragments (Fig. 5.3, tracks 4 and 6). Clones C9, D7, E3 and D3 were used to infect calves 114, 112, 115 and 123 respectively (C.G.D.Brown, unpublished data). Macroschizont-infected cell lines were established from these calves and used to

**Figure 5.3** Southern blot of *T.annulata*-infected cell lines digested with *Eco*RI and hybridised with probe *Ta*T 16. Tracks correspond to stocks.

Tracks	<i>T.annulata</i> -infected cell lines
1	<i>T.a</i> Ankara
2	<i>T.a</i> Ankara clone C9
3	<i>T.a</i> Ankara 114
4	<i>T.a</i> Ankara clone E3
5	<i>T.a</i> Ankara 115
6	<i>T.a</i> Ankara clone D3
7	<i>T.a</i> Ankara 123
8	<i>T.a</i> Ankara D7
9	<i>T.a</i> Ankara 112
10	<i>T.a</i> Hissar
11	<i>T.a</i> Hissar clone A1
12	<i>T.a</i> Hissar clone A6
13	<i>T.a</i> Hissar clone B1
14	<i>T.a</i> Hissar clone A12
15	<i>T.a</i> Gharb
16 - 19	<i>T.a</i> Gharb cloned cell lines



prepare DNA extracts. The genomic analysis of these cell lines, using probe *TaT* 16, showed that their profile did not differ from the original clones. Compare: tracks 2 and 3 (C9/114); Tracks 4 and 5 (E3/115); Tracks 6 and 7 (D3/123) and Tracks 8 and 9 (D7/112) in Fig. 5.3.

In the *T.annulata* Hissar stock only the 6.5Kb fragment was observed (Fig. 5.3, track 10) whereas clones derived from the *T.annulata* Hissar stock presented two different profiles. Clones A1, A6 and B1 showed the 6.5Kb and 3.2Kb fragments (Fig. 5.3, tracks 11, 12, 13) whereas clone A12 showed four fragments of 5.7Kb, 3.2Kb, 1.2Kb and 0.9Kb (Fig. 5.3, track 14). The *T.annulata* Gharb stocks showed a profile with only one fragment at 6.5Kb (Fig. 5.3, track 15).

In conclusion, probe *TaT* 16, showed polymorphism between different stocks isolated in Tunisia and other countries. It showed the presence of mixed genotypes in the same isolate which segregated upon cloning by limiting dilution of the cells. It did not detect any changes in clones passaged through animals but the profiles observed in piroplasms were significantly more complex than the homologous schizont-infected cell lines.

### 5.3.2 Probe *TaT* 17.

The DNA preparations from the piroplasm stocks illustrated in Fig. 5.1 were hybridised using the same blot with probe *TaT* 17 (Fig. 5.4). On *Eco*RI digests, six different profiles were observed in the six DNA preparations composed of five fragments of 7.6Kb, 6.8Kb, 5.6Kb, 4.9Kb and 1.8Kb in size. All five fragments were present in stocks *TaTu* 9A (the 7.6Kb band is not visible on the figure) and 9B (tracks 4 and 5) while stock *TaTu* 2/029 (track 3) only contained the 6.8Kb, 4.9Kb and 1.8Kb fragments and stock *TaTu* 1/270 (track 1) the 4.3Kb and 1.8Kb fragments. Both stocks *TaTu* 7 (track 2) and *TaTu* 10 (track 6) contained unique bands of 7.6Kb and 6.8Kb respectively. The *Hinf* I digests showed mainly four fragments of 1.6Kb, 1.4Kb, 1.1Kb and 0.5Kb. The 1.1Kb fragment was only present in stocks *TaTu* 7 (track 8) and *TaTu* 2/029 (not visible on the figure).

Fig. 5.5 shows a Southern blot of DNA piroplasms from *T.annulata*, isolated from Tunisia and other countries, and from *T.parva* Muguga from Kenya. An additional fragment of 8.6Kb not present in the other Tunisian stocks examined (Fig. 5.4) was detected as a

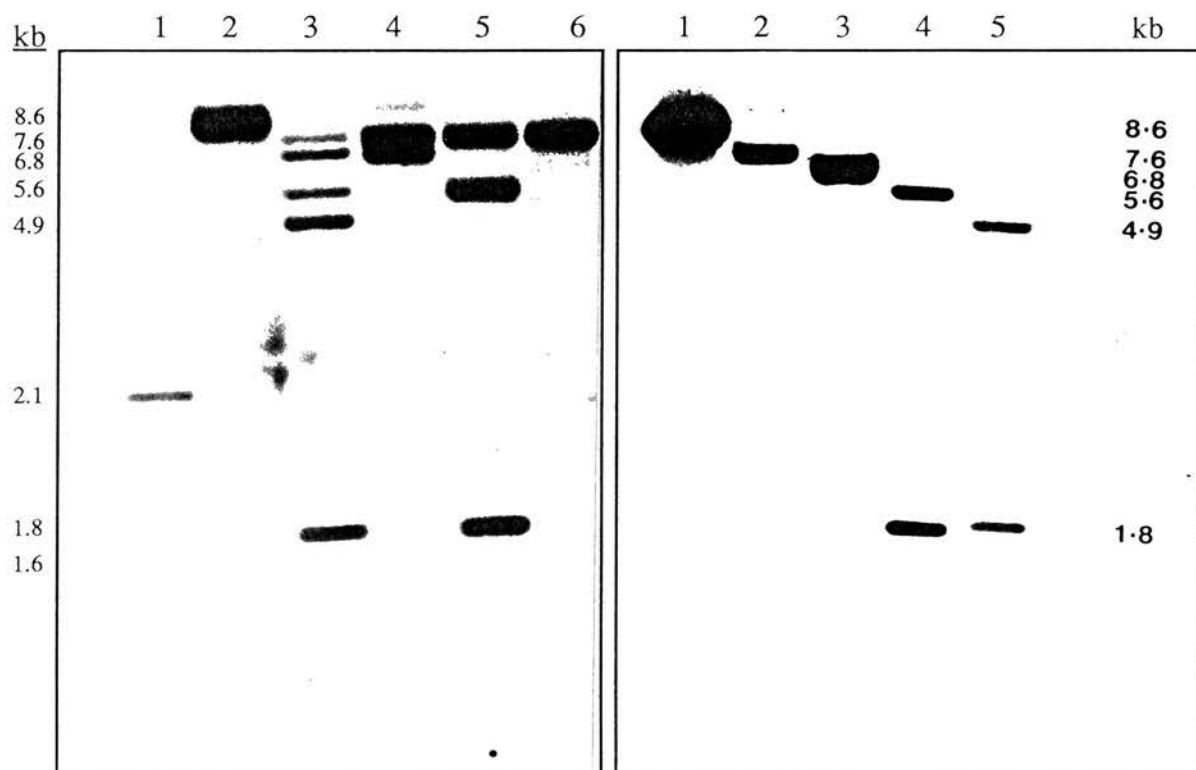
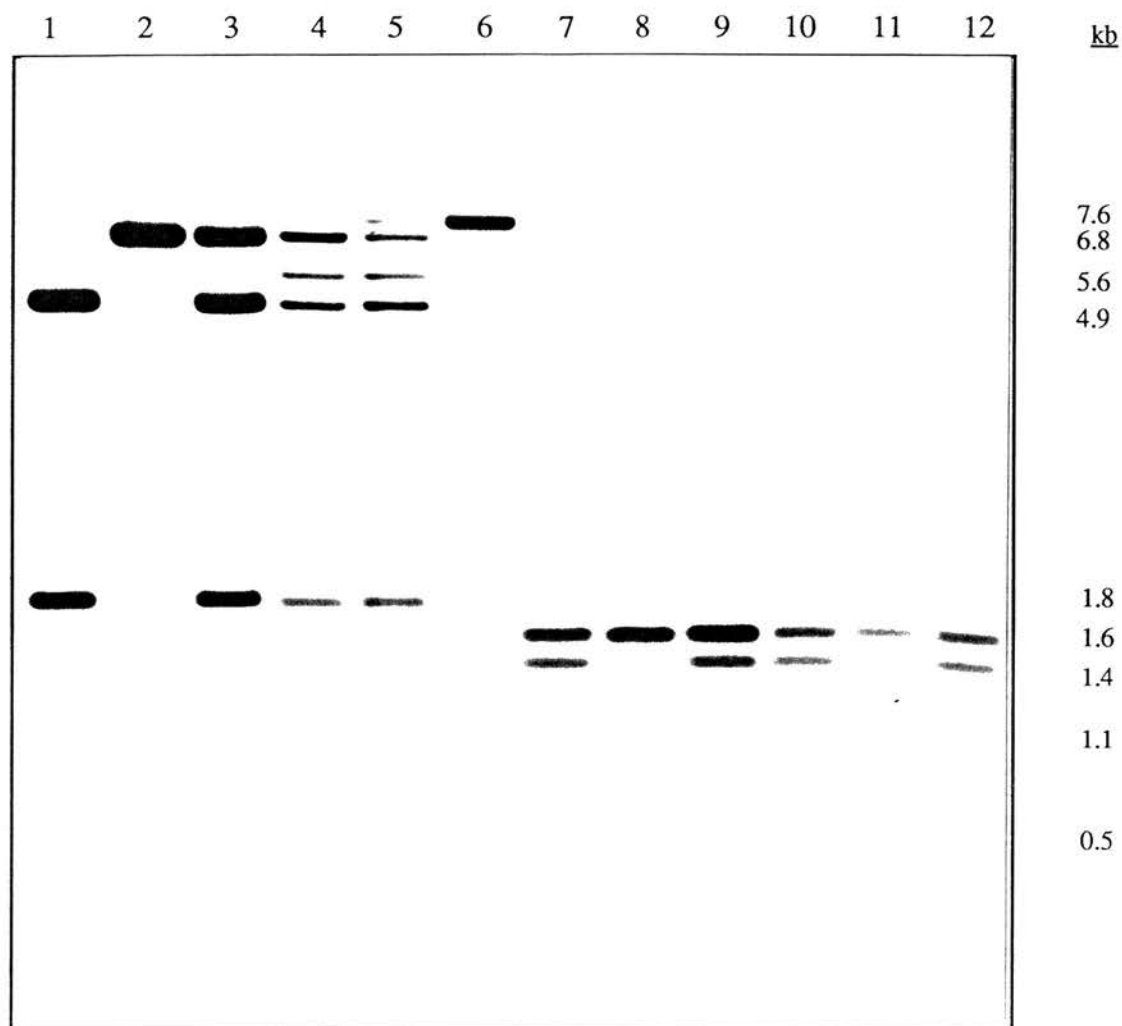


**Figure 5.4** Southern blot of *T.annulata* piroplasms digested with *Eco*RI (tracks 1-6) and *Hinf* I (tracks 7-12), and hybridised with probe *TaT* 17. Tracks correspond to strocks listed in Fig. 5.1.

**Figure 5.5** Southern blot of Theileria piroplasms digested with *Eco*RI and hybridised with probe *TaT* 17. Tracks correspond to stocks.

**Figure 5.6** Southern blot of cloned *T.annulata*-infected cell lines digested with *Eco*RI and hybridised with probe *TaT* 17. Tracks corespond to clones.

Tracks	<i>Theileria</i> piroplasms	Tracks	<i>T.annulata</i> clones
1	<i>T.parva</i> Muguga	1	1
2	<i>Ta</i> Tu 13	2	2
3	<i>Ta</i> Tu 9B	3	3
4	<i>Ta</i> Ankara	4	4
5	<i>Ta</i> Hissar	5	5
6	<i>Ta</i> Gharb		



unique band in stock *TaTu* 13 (Fig. 5.5, track 2) and together with the 7.6Kb and 6.8Kb fragments in *T.annulata* Ankara (track 4). The *T.annulata* Hissar stock showed three fragments of 7.6Kb, 5.8Kb and 1.8Kb (track 5) whereas the *T.annulata* Gharb stock (track 6) showed a single fragment of 7.6Kb. Two fragments of 2.1Kb and 1.6Kb were detected in the DNA of *T.parva* Muguga piroplasms, the 1.6Kb hybridising less intensely (track 1).

In order to define whether these multiple fragments represented a multiple copy gene or were due to mixed parasite genotypes, cloned cell lines were hybridised with the *TaT* 17 probe. Fig. 5.6 illustrates the five profiles found in these clones. Clone 1 derived from stock *TaTu* 13, clones 2 and 3 from stock *TaTu* 9B, clone 4 from stock *TaTu* 9A and clone 5 from stock *TaTu* 2. They are referred to in this study as clones 1-5. They were selected as representatives of the five genotypes identified by probe *TaT* 17. Clones 1, 2 and 3 showed unique fragments of 8.6Kb, 7.6Kb and 6.8Kb respectively whereas clones 4 and 5 showed a common 1.8Kb fragment, due to an extra *EcoRI* restriction site, in addition to a second fragment of 5.6Kb and 4.9Kb respectively. Thus this probe detects a single copy gene which is highly polymorphic between the Tunisian isolates. Clones of *T.annulata* Ankara were all identical when Southern blots were probed with *TaT* 17 and showed a single fragment of 7.6Kb. Similarly *T.annulata* Hissar cloned cell lines were also identical but showed the same profile detected in the parental cell line (5.6Kb/1.8Kb) (data not shown).

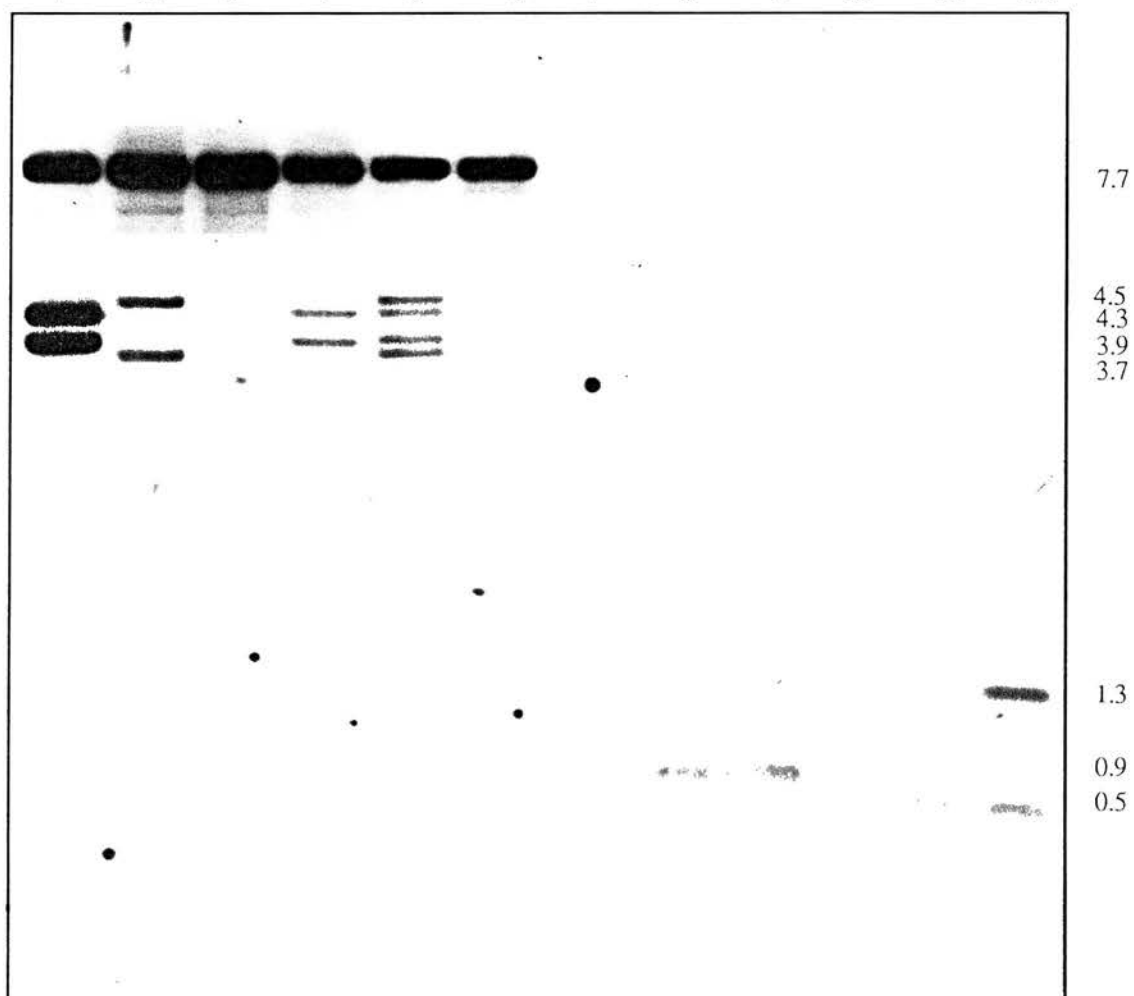
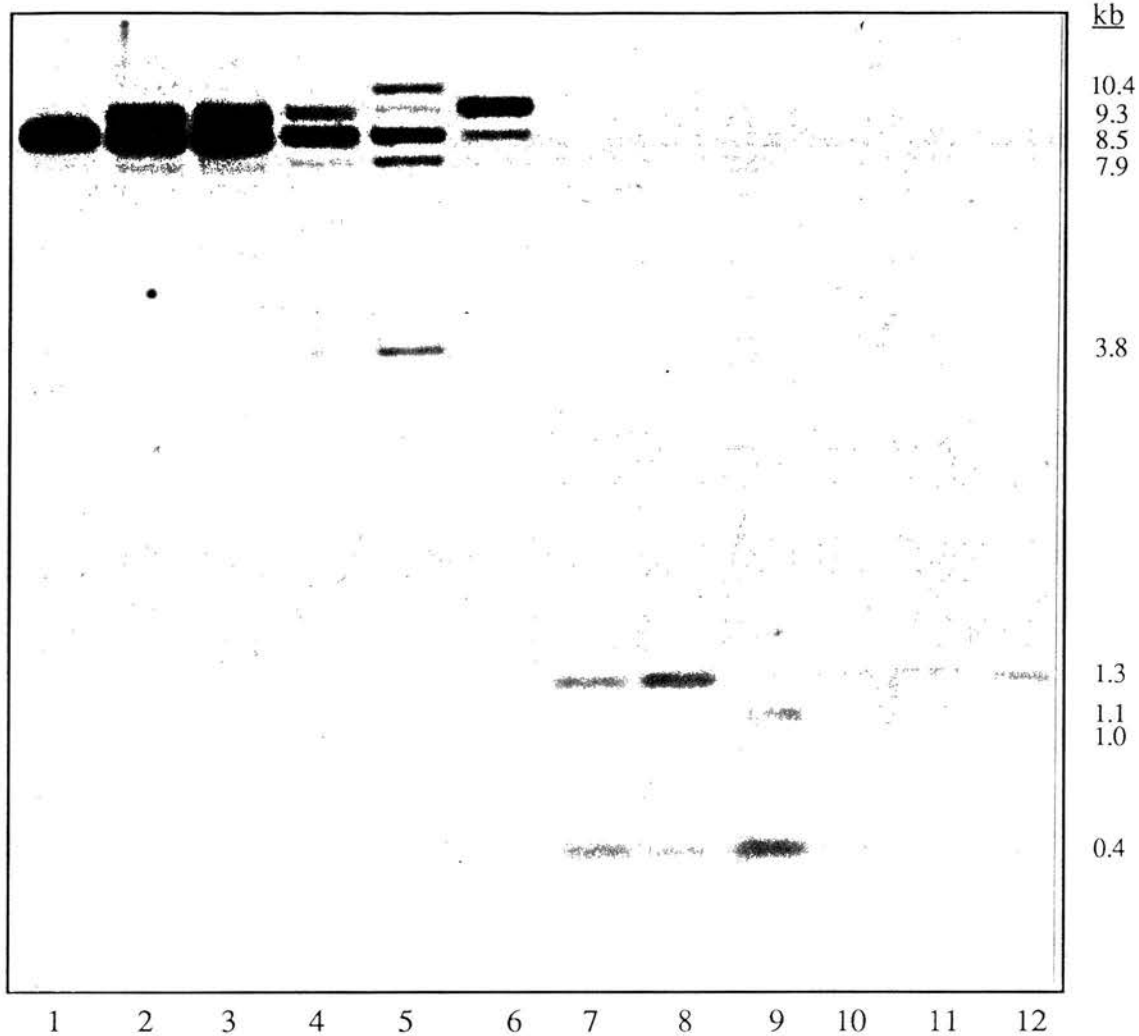
### 5.3.3 Probe *TaT* 20.

This probe detected five fragments of different size and four different profiles in the DNA of the standard panel of stocks digested with *EcoRI* (Fig. 5.7). Hybridisation to *Hinf* I digests of DNA from the same stocks showed three different profiles composed of four fragments with three of the stocks showing the presence of two fragments (Fig. 5.7).

In the *EcoRI* profiles, one fragment of 8.5Kb was present in all stocks, although the intensity varied between stocks. The 9.2Kb fragment was not detected in stock *TaTu* 1/270 (track 1) and was faint in stock *TaTu* 9B (track 5), which also showed a 10.4Kb fragment and two fragments of 7.9Kb and 3.8Kb respectively. These two fragments were also present in stock *TaTu* 9A but showed a faint hybridisation signal (track 4).

**Figure 5.7** Southern blot of *T.annulata* piroplasms digested with *Eco*RI (tracks 1-6) and *Hinf* I (tracks 7-12), and hybridised with probe *TaT* 20. Tracks correspond to stocks listed in Fig. 5.1.

**Figure 5.8** Southern blot of *T.annulata* piroplasms digested with *Eco*RI (tracks 1-6) and *Hinf* I (tracks 7-12), and hybridised with probe *TaT* 21. Tracks correspond to stocks listed in Fig. 5.1.



Southern blots of *Hinf* I digests of the DNA from the same stocks were probed and four fragments of 1.3Kb, 1.1Kb, 1Kb and 0.4Kb detected. The 1.1Kb fragment was only present in stock *TaTu* 2/029 (track 9). Stocks *TaTu* 9A, 9B and 10 (tracks 10, 11 and 12) showed an additional fragment of 1Kb compared to stocks *TaTu* 1/270 (track 7) and *TaTu* 7 (track 8) which had two fragments of 1.3Kb and 0.4Kb. The 0.4Kb band was detected in all stocks.

#### **5.3.4 Probe *TaT* 21.**

The same stocks were analysed by Southern blotting of *Eco*RI and *Hinf* I digests using probe *TaT* 21. The *Hinf* I pattern was identical for five of the stocks tested and was composed of fragments of small size hybridising to the probe. Stock *TaTu* 10 showed an additional fragment which hybridised strongly with *TaT* 21 (Fig. 5.8, track 12). Fig. 5.8 also shows the profile patterns observed on *Eco*RI digests from DNA probed with *TaT* 21. Five fragments of 7.7Kb, 4.5Kb, 4.3Kb, 3.9Kb and 3.7Kb were observed. The five clones, as defined by probe *TaT* 17, showed three different profiles when their DNA was hybridised with probe *TaT* 21 (Fig. 5.9). Clones 2, 3 and 4 showed only the 7.7Kb fragments and clone 1 the 4.3Kb and 3.9Kb fragments whereas clone 5 showed the three fragments together (Fig. 5.9).

Fig. 5.10 shows the hybridisation profiles obtained with probe *TaT* 21 on DNA of *T.annulata* stocks from other countries and *T.parva* Muguga. Both *T.annulata* Ankara and Hissar showed identical profiles already detected in the Tunisian stocks and composed of the five fragments (Fig. 5.10, tracks 4, 5). *T.annulata* Gharb however showed a profile identical to *TaTu* 13 and to clone 1 and composed of the two fragments of 4.3Kb and 3.9Kb. *T.parva* Muguga did not show any hybridisation signal with probe *TaT* 21 (Fig. 5.10, track 1).

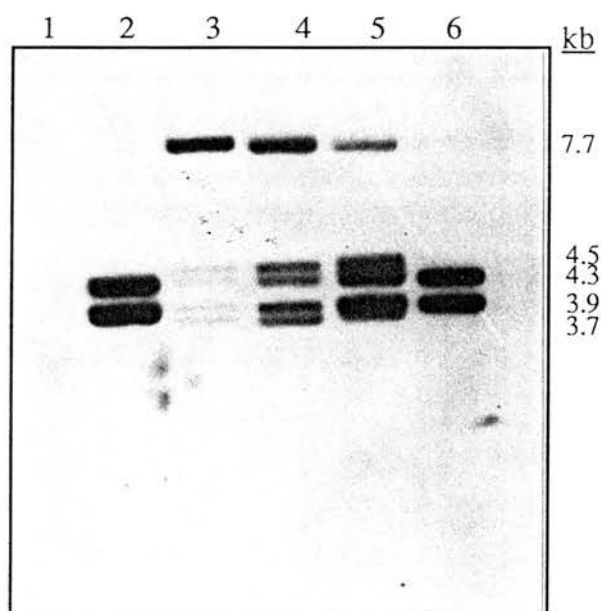
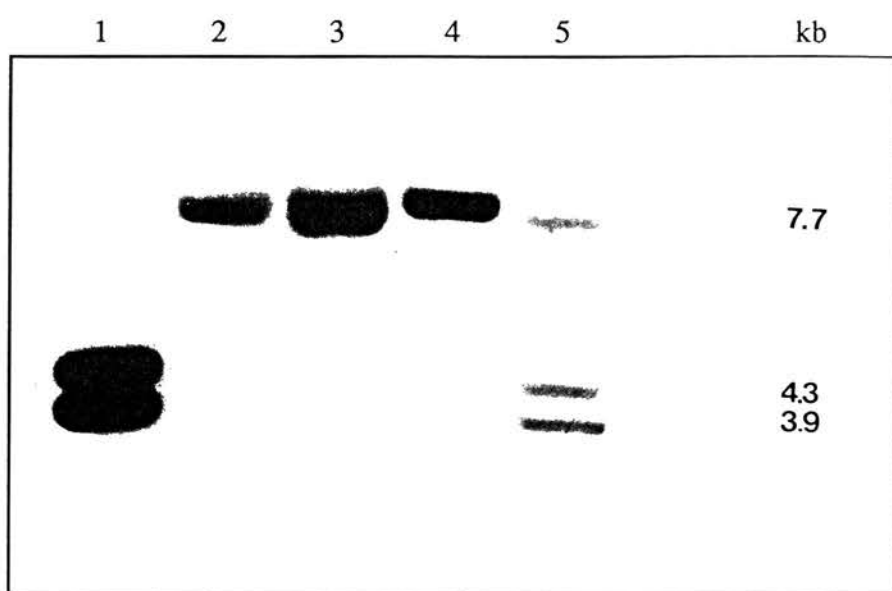
#### **5.3.5 Additional probes examined.**

In order to find if the polymorphism detected in Tunisian isolates is also observed with probes selected from genomic libraries of *T.annulata* parasites isolated from other countries, two gene probes were used to hybridise DNA from various stocks. These were the sporozoite antigen gene probe (SPAG-1) (Williamson *et al*, 1989) and the merozoite rhoptry antigen

**Figure 5.9** Southern blot of cloned *T.annulata*-infected cell lines digested with *Eco*RI and hybridised with probe *Ta*T 21. Tracks correspond to clones listed in Fig. 5.6

**Figure 5.10** Southern blot of *Theileria* piroplasms digested with *Eco*RI and hybridised with probe *Ta*T 21. Tracks correspond to stocks listed in Fig. 5.5.





gene probe (E2.1) (B.R.Shields, unpublished data) both selected from a *T.annulata* Ankara genomic library.

Southern blots of *Eco*RI digests of genomic DNA from Tunisian *T.annulata* stocks and clones were probed with SPAG-1. Two fragments of 5.7Kb and 4.6Kb were observed (Fig. 5.11). The study of Williamson *et al* (1989) on *T.annulata* Ankara and *T.annulata* Hissar DNA using the same probe showed the presence of three fragments of 6Kb, 4.8Kb and 3.4Kb. The first two fragments, although showing slightly different sizes, seems to relate to the 5.7Kb and 4.6Kb fragments detected in the Tunisian stocks. The 3.4Kb fragment or a fragment of approximately the same size was not observed in these stocks.

The E2.1 gene probe identified two fragments of 4Kb and 2.8Kb when hybridised with DNA extracts from Tunisian stocks. These are illustrated in Fig. 5.12. These fragments were observed either together or individually (Fig. 5.12, tracks 2 and 3). The same probe detected an additional fragment of 3.3Kb in *T.annulata* Hissar and *T.annulata* Gharb (Fig. 5.12, tracks 5 and 6).

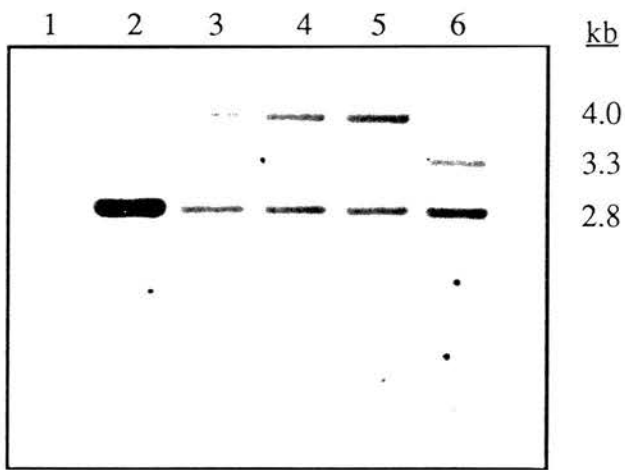
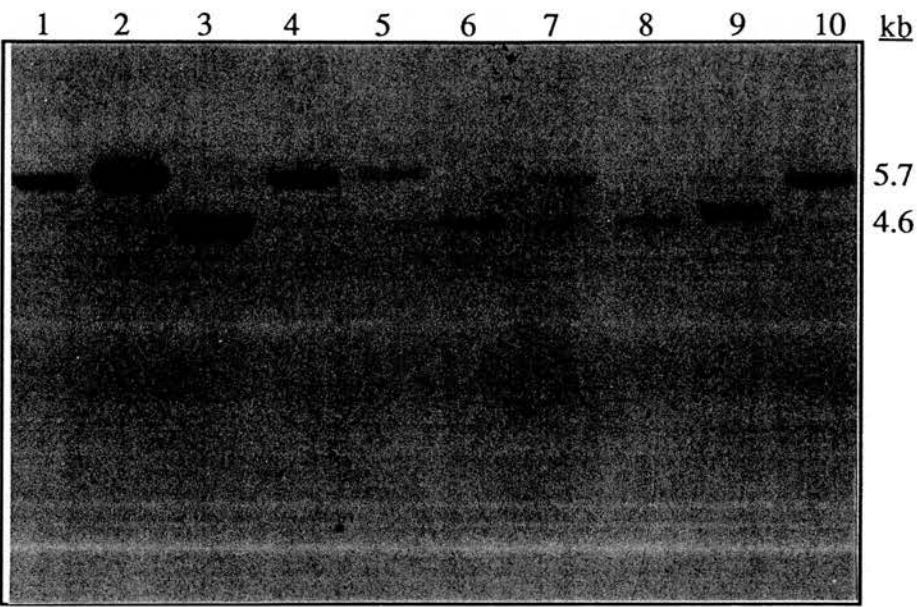
### 5.3.6 Subcloning and sequencing of *TaT 17* probe.

Probe *TaT 17* was selected as the most suitable probe to study the polymorphism in the Tunisian *T.annulata* stocks. Therefore a study on the specific fragments responsible for the polymorphism observed was carried out. Of five derived fragments from probe *TaT 17*, two of 0.7Kb and 1Kb were subcloned in a phagemid for sequence studies in order to determine any homology with *T.annulata* and other protozoan parasite gene sequences already known in the data base.

Eight restriction enzymes listed in Fig. 5.13, were used to digest pUC18 recombinant plasmid containing the *TaT 17* insert probe of 6.8Kb as per manufacturer's recommendations (BRL). The *Eco*RI digest gave the predicted plasmid fragment of approximately 2.7Kb and the insert fragment, *TaT 17* of 6.8Kb (Fig. 5.13, track 1). A similar pattern was obtained with the *Xba* I digest (Fig. 5.13, track 5). The *Bam* HI digest was represented by a large fragment of approximately 5Kb and another fragment of approximately 3.4Kb (Fig. 5.13, track 2). Similarly two fragments of approximately 7.5Kb and 1.8Kb were observed in *Xho* I digests

**Figure 5.11** Southern blot of 10 Tunisian *T.annulata*-infected cell lines digested with *Eco*RI and hybridised with probe **SPAG-1**.

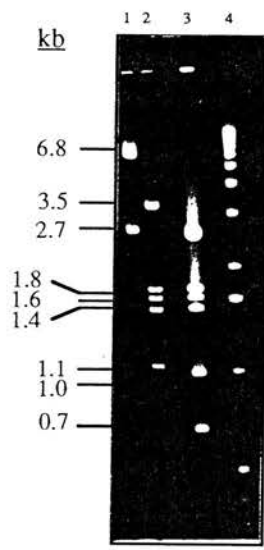
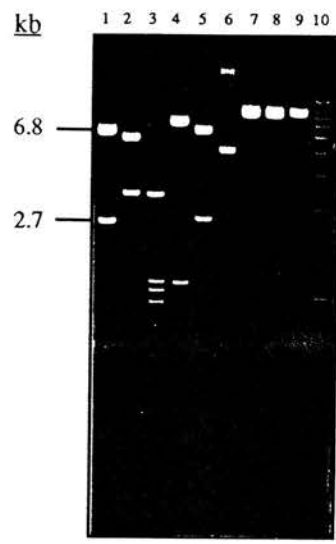
**Figure 5.12** Southern blot of *Theileria* piroplasms digested with *Eco*RI and hybridised with probe **E2.1**. Tracks correspond to stocks listed in Fig. 5.5.



**Figure 5.13** Agarose gel, stained with ethidium bromide, of pUC 18 containing the 6.8Kb insert and digested with nine restriction enzymes. Tracks correspond to restriction digests from each enzyme (Molecular markers are shown in track 10).

Tracks	Enzyme
1	<i>Eco</i> RI
2	<i>Bam</i> HI
3	<i>Hind</i> III
4	<i>Xho</i> I
5	<i>Xba</i> I
6	<i>Sac</i> I
7	<i>Sma</i> I
8	<i>Sal</i> I
9	<i>Pst</i> I

**Figure 5.14** Agarose gel, stained with ethidium bromide, of pUC 18 with the 6.8Kb insert and digested with *Eco*RI alone (track 1), *Hind* III alone (track 2) and *Eco*RI and *Hind* III (track 3). (Molecular markers are shown in track 4).



(Fig. 5.13, track 4). Only one fragment was obtained with *Sac* I (the upper band corresponds to undigested DNA), *Sma* I, *Sal* I and *Pst* I digests (Fig. 5.13, tracks 6-9). The *Hind* III digest was composed of five fragments detected on the ethidium bromide gels (Fig. 5.13, track 3 and Fig. 5.14, track 2). They were of approximately 3.5Kb, 1.8Kb, 1.6Kb, 1.4Kb and 1.1Kb. These fragments were the most suitable for subcloning the *TaT* 17 probe. In order to isolate purified fragments from this probe without the plasmid DNA, a double digestion with both *Eco*RI and *Hind* III was performed. As a consequence six fragments were obtained of approximately 2.7Kb, 1.8Kb, 1.6Kb, 1.4Kb, 1Kb and 0.7Kb (Fig. 5.14, track 3). The differences observed between the *Hind* III profile and the *Eco*RI/*Hind* III profile of *TaT* 17 digests was shown by the appearance of three fragments of 2.7Kb, 1Kb and 0.7Kb and the disappearance of the 3.5Kb and 1.1Kb fragments initially detected on the *Hind* III digests. Since probe *TaT* 17 was first inserted in the plasmid using the only *Eco*RI site in the polycloning linker (PCS) of pUC 18, the 2.7Kb fragment corresponds to the plasmid itself and the other fragments, to the inserted sequence of *TaT* 17 insert with *Hind* III. Hence the *TaT* 17 insert contained four restriction *Hind* III sites giving rise to five fragments; the sixth fragment observed in the ethidium bromide gel in Fig. 5.14 (track 3) being the plasmid (2.7Kb). In *Hind* III digests of the recombinant plasmid probe, one insert fragment of 0.7Kb, remained attached to the plasmid. This was isolated from the plasmid by the double digestion with both *Eco*RI and *Hind* III. The fragment size of the 2.7Kb and 0.7Kb when added together correspond approximately to the 3.5Kb fragment (compare in Fig. 5.14, tracks 2 and 3). Therefore the 0.7Kb fragment derived from the 3.5Kb fragment detected, represents one of the probe extremities and has a *Hind* III restriction site at one end and the *Eco*RI restriction site at the other. For convenience, the *Eco*RI site on the 0.7Kb fragment, will be referred to as the right hand side of the insert and the other extremity, limited by the other *Eco*RI restriction site, as the left hand side. The 1.1Kb fragment observed on *Hind* III digests corresponds almost certainly to the 1Kb fragment observed on the *Eco*RI/*Hind* III digests. The former fragment being flanked by two *Hind* III sites whereas the latter being flanked by an *Eco*RI site one end and a *Hind* III site on the other end. The difference in size detected between the two fragments was approximately 0.1Kb. Therefore the *Eco*RI site was very close to one of the



*Hind* III sites and this *Hind* III site belongs to the PCS of the plasmid, since the *Eco*RI site limits the probe. The pUC 18 PCS is limited by *Eco*RI on one side and by *Hind* III on the other side at 0.06Kb. The fact that by using *Hind* III the fragment obtained is slightly larger suggests that this fragment is located at the left hand side of the probe next to the PCS of the plasmid. The difference in size between the two fragments represented by the PCS of the plasmid would not be detectable by gel electrophoresis because of its small size (0.06Kb). A putative scheme representing the plasmid and the fragments obtained after digestion with both *Eco*RI and *Hind* III is given in Fig. 5.15.

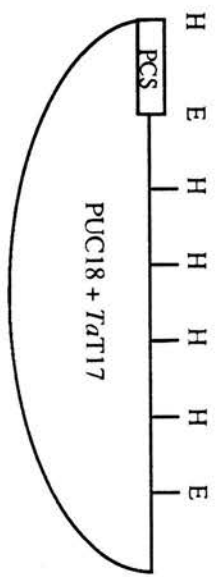
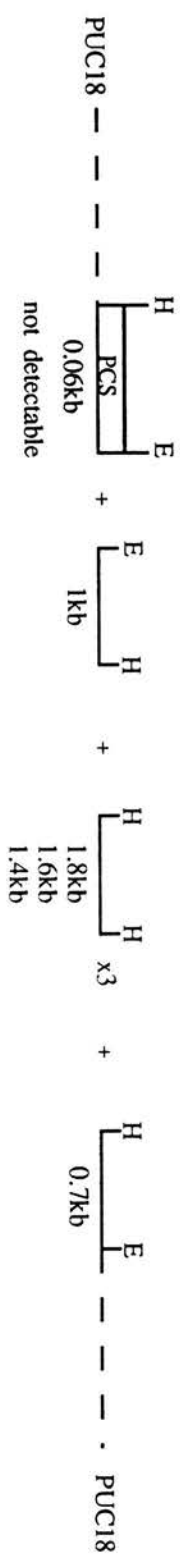
Fragments (F) 1 and 2 of 0.7Kb and 1Kb respectively were isolated from the agarose gel, purified using a commercial kit (Gene clean) and ligated into phagemid pBluescript II SK. The latter was then used to transform competent *E.coli*. Both F1 and F2 were sequenced using primers for the T3 and T7 promoters located outside the insert on the phagemid. Up to 300bp were sequenced for each fragment from both insert sites in the phagemid. The sequences obtained were used to screen for homology using the data base. The only homology identified was with a cysteine proteinase gene isolated from *T.parva* (Nene *et al*, 1990). This sequence was read from the T3 promoter (*Eco*RI terminal end) on the 1Kb (F2) fragment and the deduced amino acid sequence compared with the *T.parva* cysteine proteinase (Fig. 5.16). They showed 42.647% similarity. The sequence read from the T7 promoter (*Hind* III terminal end), did not show any homology with any sequence in the data base, nor did either of the sequences of the 0.7Kb fragment (data not shown).

#### **5.3.7 Restriction map of the fragment detected by probe TaT 17 in the five clones.**

An attempt was made to explain the restriction fragments detected by probe TaT 17 in the Tunisian stocks. This was conducted using individual cloned cell lines representative of the five populations detected by the probe.

The five fragments (F1-F5) obtained from the double digestion of probe TaT 17 with *Eco*RI and *Hind* III of, respectively 0.7Kb, 1Kb, 1.4Kb, 1.6Kb and 1.8Kb, were isolated from low melting agarose gels and used to probe Southern blots of restriction digests of genomic DNA from the five clones (Fig. 5.17). Clone 1 (tracks 1-3), clone 2 (tracks 4-6), clone 3 (tracks 7-9), clone 4 (tracks 10-12) and clone 5 (tracks 13-15) were digested with *Eco*RI

**Figure 5.15** Representative scheme of double digests of probe *TaT* 17 with *Eco*RI(E) and *Hind* III (H) and their putative localisation within pUC 18.



**Figure 5.16** Comparison between part of the amino acid sequence of the *T.parva* cysteine proteinase (A), and the partial protein sequence deduced from F2 (TaT 17) nucleotide sequence (B).

*T.p:* *T.parva* cysteine proteinase. Sequence given by nucleotides residues 1330-992 (Nené *et al*, 1990).

*T.a:* *T.annulata* F2 protein. Sequence given by nucleotides residues 1-343 (F2).

<i>T.p</i>	V	V	W	Y	R	K	K	T	V	E	D	Y	G	E
<i>T.a</i>	I	I	W	Y	R	K	K	L	T	D	D	Y	G	E
<i>T.p</i>	G	V	L	V	V	A	H	N	L	S	K	G	C	E
<i>T.a</i>	G	V	L	L	V	A	H	N	L	E	Q	A	C	E
<i>T.p</i>	G	T	F	V	G	S	K	Y	K	A	L	E	P	S
<i>T.a</i>	G	N	Y	I	G	S	K	Y	M	T	L	D	Y	S
<i>T.p</i>	V	S	L	Y	V	S	C	P	S	S	L	T	S	R
<i>T.a</i>	S	A	I	Y	L	I	T	P	S	L	V	I	S	K
<i>T.p</i>	T	M	V	E	K	G	K	F	V	H	Y	S	P	V
<i>T.a</i>	N	L	I	D	L	G	S	S	I	T	F	Y	N	I
<i>T.p</i>	S	V	K	K	A	K	P	V	S	C	R	R	A	K
<i>T.a</i>	Y	T	K	K	T	S	K	V	V	C	T	N	K	K
<i>T.p</i>	D	V	F	P	L	D	K	A	S	V	L	G	Y	K
<i>T.a</i>	A	L	M	L	Y	I	N	I	N	S	V	G	K	N
<i>T.p</i>	R	V	Y	E	Y	A	S	E	L	L	G	G	Q	C
<i>T.a</i>	Q	I	Y	Q	L	A	T	N	G	F	G	G	E	C
<i>T.p</i>	G													
<i>T.a</i>	G													

Percentage of similarity: 42.647

alone (tracks 1, 4, 7, 10 and 13), *Eco*RI and *Hind* III (tracks 2, 5, 8, 11 and 14) and *Hind* III alone (tracks 3, 6, 9, 12 and 15).

When the blots were hybridised with probe F2 (1Kb) some of the fragments observed were common to all clones whereas, others were present in only some of the clones. All clones showed 4.2Kb and 1.8Kb fragments in *Hind* III digests and a 1Kb fragment in *Eco*RI/*Hind* III digests. A 1.8Kb fragment was also observed in *Eco*RI/*Hind* III digests of clones 1, 2 and 3 and in *Hind* III digests of clones 4 and 5 (Fig. 5.17 A).

The DNA extracts from the five clones digested with *Eco*RI and probed with F2, F3 and F5 (Fig. 5.17 A, C, B) presented a similar pattern to the profiles observed when these clones were probed with the whole *TaT* 17. (As described in Fig. 5.6).

Probe F5 (1.8Kb) (Fig. 5.17 B) showed the same profiles seen with F2 probe but the 4.2Kb and 1Kb bands were fainter. In addition clone 4 showed a 0.8Kb band and two fainter bands of 1.4Kb and 1.6Kb in *Eco*RI/*Hind* III digests. The latter were also seen in the *Hind* III digest of the same clone.

Probe F3 (1.4Kb) (Fig. 5.17 C) hybridised to a 1.4Kb fragment in all *Eco*RI/*Hind* III digests and *Hind* III digests. It also hybridised to the 1.8Kb band seen on F2 and F5 blots but the signal given was not as strong. This probe detected the 0.8Kb and 1Kb bands in extracts from clone 4 and clone 5 digested with *Eco*RI/*Hind* III, similarly to F5, but the reactivity with the latter was very faint.

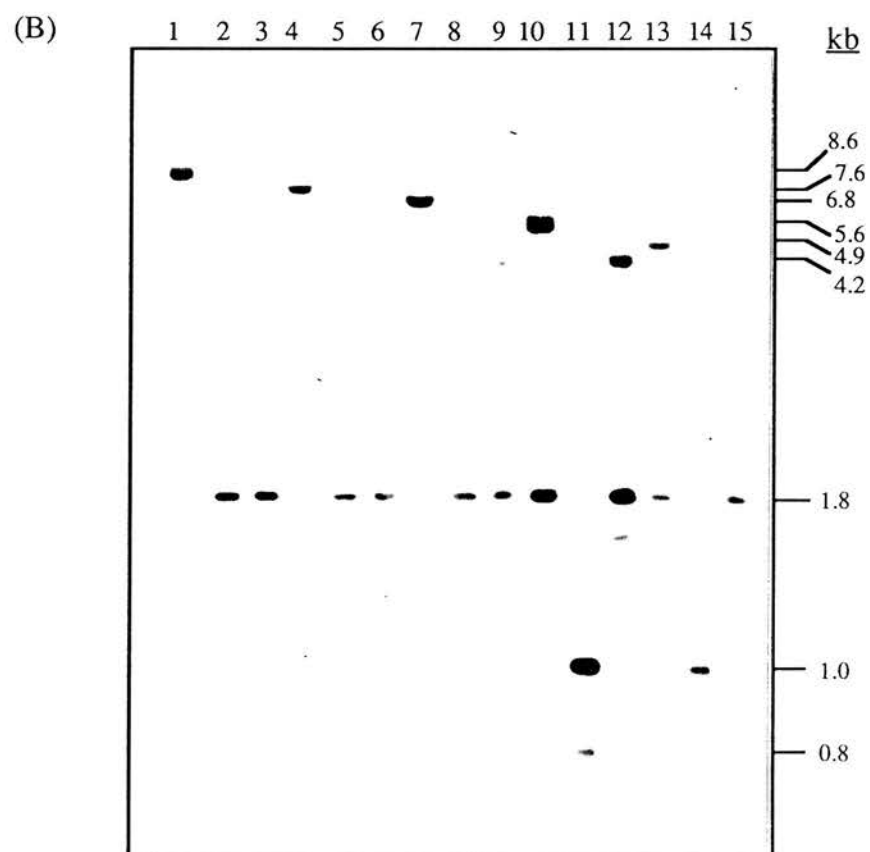
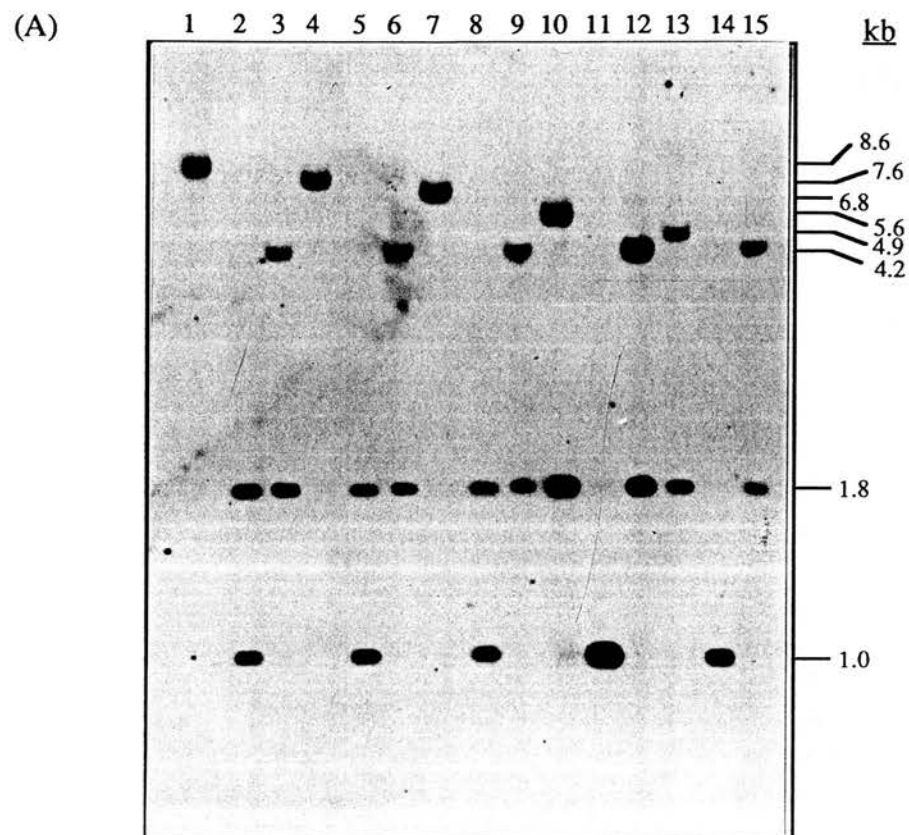
Probe F4 (1.6Kb) (Fig. 5.17 D) identified a 1.6Kb band in all *Eco*RI/*Hind* III digests and *Hind* III digests. In *Eco*RI digests it showed the 8.6Kb band in clone 1, the 7.6Kb band in clone 2, the 6.8Kb band in clone 3, the 5.6Kb band in clone 4 and the 4.9Kb band in clone 5. A faint band of 1.4Kb was also seen in *Eco*RI/*Hind* III digests of clone 4.

Probe F1 (0.7Kb) (Fig. 5.17 E) recognised a 8.3Kb band in all *Hind* III digests and a similar profile to F4 in *Eco*RI digests. Two additional fragments were detected by probe F1 in *Eco*RI/*Hind* III digests. These were a 1.2Kb band in clones 2 and 4 and a 0.7Kb band in clones 1, 3 and 5.

Hence, the five fragments detected specific bands as well as common bands observed on the five blots. The former would help in identifying the position of each fragment in the

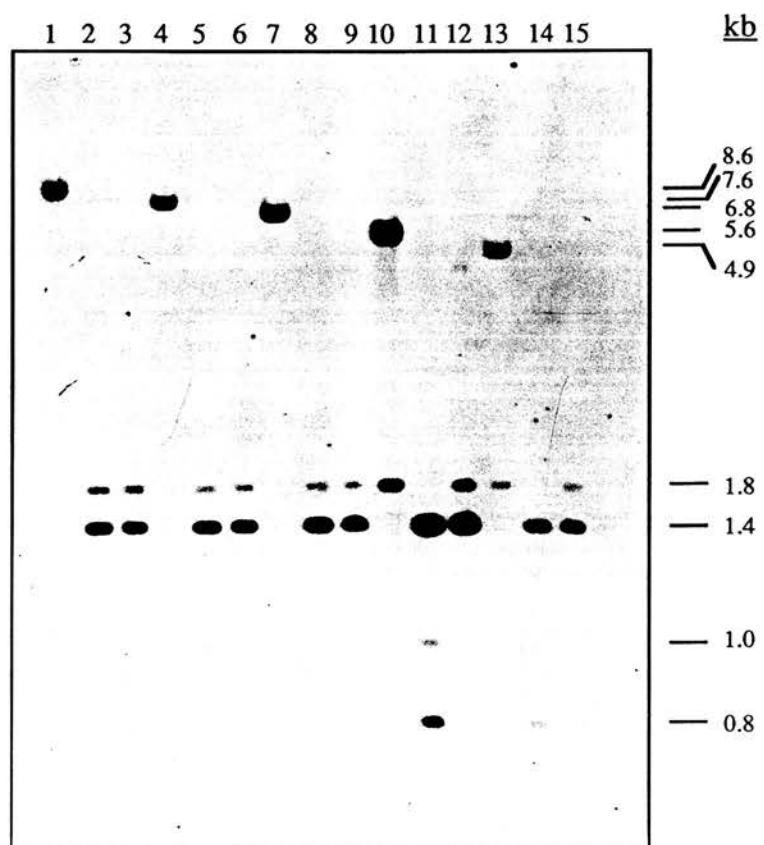
**Figure 5.17** Southern blot of the five cloned *T.annulata*-infected cell lines (Clones 1-5) digested with *Eco*RI alone (tracks 1, 4, 7, 10 and 13), *Eco*RI and *Hind* III (tracks 2, 5, 8, 11 and 14) and *Hind* III alone (tracks 3, 6, 9, 12 and 15). Tracks correspond to clones. Clone 1 (tracks 1, 2 and 3), clone 2 (tracks 4, 5 and 6), clone 3 (tracks 7, 8 and 9), clone 4 (tracks 10, 11 and 12) and clone 5 (tracks 13, 14 and 15). Hybridisation of the blot were carried out with fractions (F) 1-5 and are presented as follows:

Fig. 5.17 A	F2 (1 Kb)
Fig. 5.17 B	F5 (1.8 Kb)
Fig. 5.17 C	F3 (1.4 Kb)
Fig. 5.17 D	F4 (1.6 Kb)
Fig. 5.17 E	F1 (0.7 Kb)

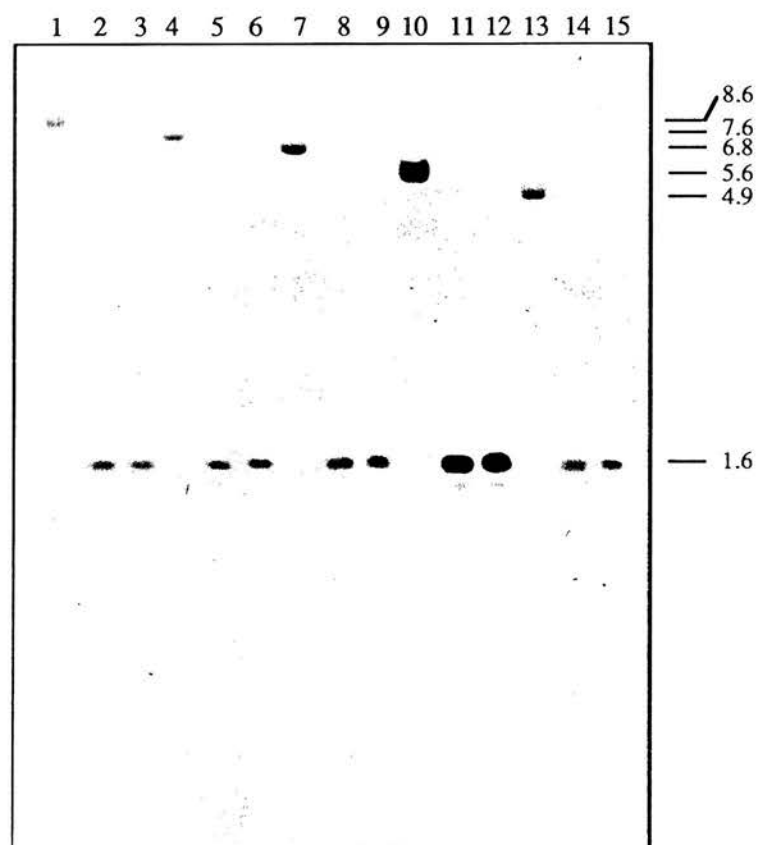


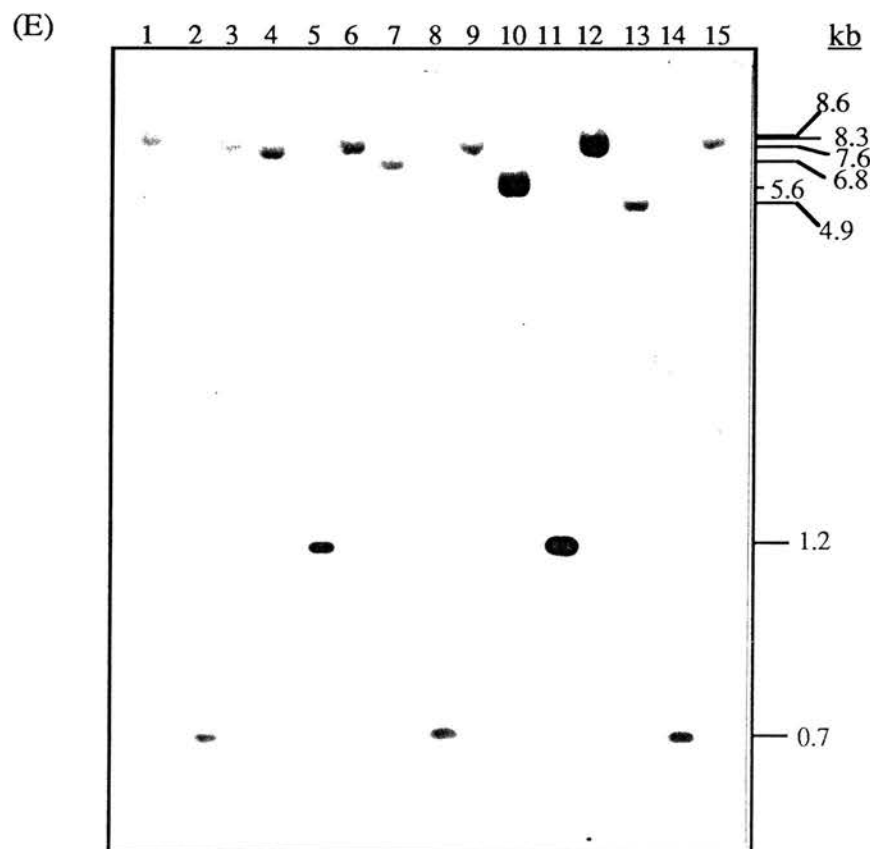


(C)



(D)





sequence recognised by probe *TaT* 17 whereas the latter would be useful to explain the polymorphism observed in the five clones.

An explanation for these results is given in the discussion. However it might not be the only possibility, but since only results from the blots with the five fragments were used to make this interpretation, it was thought to be the closest to the real map in the genome of the five clones. More accurate mapping would require isolating the homologue sequence from each clone, subcloning it in a vector and sequencing each subcloned fragment. This was not undertaken in the present study, but could be considered for future work.

#### 5.4 DISCUSSION.

The first objective of the study described in this chapter was to select a polymorphic probe for strain characterisation by analysis of the parasite genome. Such a probe would ideally represent a moderately repetitive fragment in the genome and would differentiate between different isolates. Four recombinant plasmids, containing large inserts (6.8-8.2Kb), were chosen at random to carry out the preliminary study on the Tunisian stocks. They were also shown to have single inserts which could facilitate the interpretation of the results. The results show that the four probes studied were polymorphic thus providing choice in selecting the probes to study all the Tunisian stocks. Two restriction enzymes have been used in the preliminary study, *EcoRI* and *Hinf* I. The *Hinf* I digests were less polymorphic with the four probes than the *EcoRI* digests. Therefore further studies were carried out on *EcoRI* digests of DNA from the other Tunisian stocks.

Probe *TaT* 16 showed a polymorphic banding pattern on *EcoRI* digests of the piroplasm DNA extracts but only a few of the bands were detected in DNA isolated from the schizont infected cell lines (Compare Fig. 5.1 and 5.2). The reason behind this phenomenon remains unknown. The high number of fragments detected in the piroplasm DNA digested with *EcoRI* could either be due to a high level of polymorphism of the sequences detected or to the detection of related sequences in the genome which present some homology with probe *TaT* 16, i.e. related genes. This probe showed two different profiles on piroplasm DNA derived from the same stock (1/270). Indeed the probe identified two additional bands of

0.9Kb and 1.2Kb in the pattern of the same stock as seen in Fig. 5.1. Two explanations could be put forward; either the DNA was not completely digested in the first experiment or the blotting was incomplete and the smaller fragment were not detected.

The results showed that probe *TaT* 16 detected two populations in Tunisian stocks and stocks from other countries as defined by results from cloned infected cell lines. Although this probe was able to distinguish at the genomic level between two types of clones of *T.annulata* Ankara which differed in their ability to undergo differentiation *in vitro* (Shiels *et al*, 1992), it was not selected for further studies on the Tunisian stocks because of the type of polymorphism detected, especially on piroplasm DNA.

Probe *TaT* 20 showed a polymorphic pattern amongst the Tunisian stocks, and all bands detected in piroplasm DNA were seen on schizont infected cell line DNA (data not shown). It detected at least four different populations in the stocks studied. this probe was not studied further because it only detected four profiles on the preliminary screen whereas probe *TaT* 17 detected six profiles. However the two probes produced comparable results of equal interest for the purpose of this study. As a result of this initial analysis two of the four probes were selected to study all 51 of the Tunisian isolates made in this study. These were probes *TaT* 21 and *TaT* 17.

Probe *TaT* 21 showed three profiles in the preliminary study of the Tunisian stocks. Five fragments were detected of which four occurred as two doublets. It was confirmed later in this study, with the other stocks, that each pair of doublets was always present together and segregated together in cloned cell lines as illustrated by clone 1 (Fig. 5.9). The third profile, determined by probe *TaT* 21, was composed of a fragment of 7.7Kb, as illustrated by clones 2, 3 and 4 (Fig. 5.9). Clone 5 showed both the 7.7Kb fragment and the 4.3/3.9Kb doublet. This could indicate that the cloned cell line is not a real clone or that the cloned cells were infected with two different genotypes; although the latter is unlikely. If one assumes that clone 5 is composed of two parasite genotypes, then the gene detected would be at a single locus with three different alleles resulting from the insertion of a polymorphic *Eco*RI site in two of them. Probe *TaT* 21 was used to study all the Tunisian stocks, as it was thought that a different pattern from the ones obtained with either probe *TaT* 17 or *TaT* 20 would add to the

extent of variability detected by these probes. On one occasion the *EcoRI* digestion of probe *TaT* 21 showed a doublet of approximately 7.8/8.2Kb (data not shown) and it was thought that the plasmid carried an insert of 16Kb in size with an internal *EcoRI* site. Other experiments showed that this was an artefact and that the plasmid had only one insert of approximately 8.2Kb.

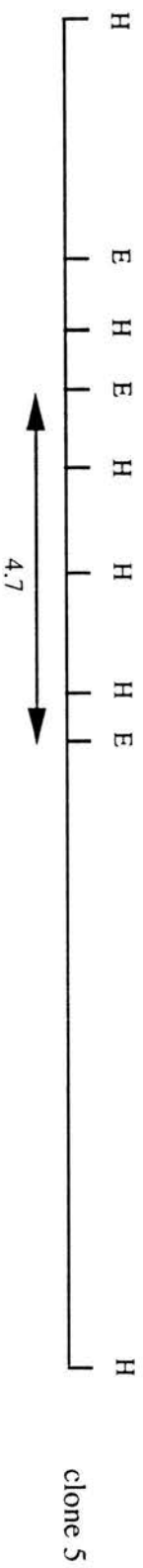
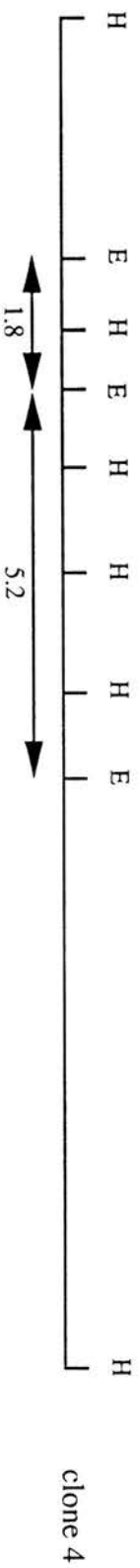
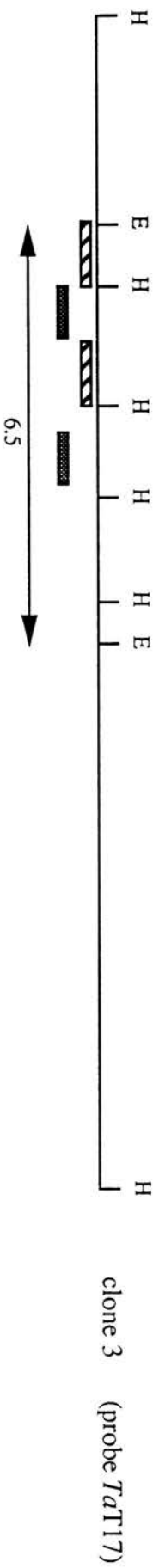
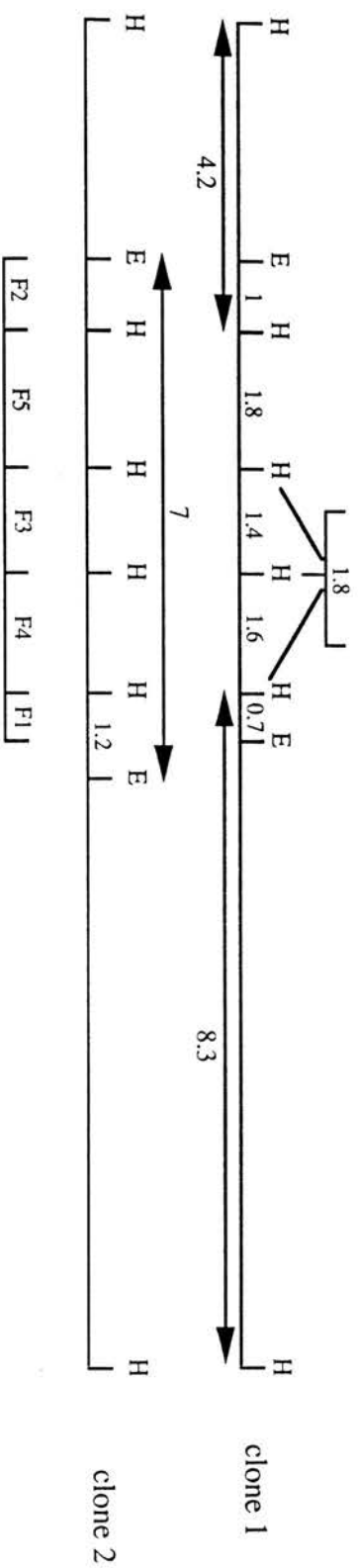
From the results obtained it is likely that probe *TaT* 17 corresponds to a single copy gene. Such a situation would be useful, so that allelic interpretation could be made, e.g. by using cloned cell lines, single alleles corresponding to single subpopulations could be identified and an overall study of the number of subpopulations present within one isolate and in different isolates could be made. The evidence that probe *TaT* 17 corresponds to a single copy gene was further supported by the hybridisation of this probe to unique fragments in some isolates (Fig. 5.4, tracks 2 and 6). This was later confirmed when cloned cell lines were used in Southern blots hybridised with probe *TaT* 17. Indeed five profiles were detected showing unique fragments for most of them. The second fragment observed in digests of two of the five clones was due to an internal *EcoRI* site within the sequence detected in these clones. Therefore probe *TaT* 17 clearly defined five subpopulations in the Tunisian stocks and some of these were observed in stocks from other countries. All bands detected in piroplasm DNA were present in macroschizont-infected cell DNA, making the results of the hybridisation profiles consistent between the two parasite stages. This probe also hybridised with *T.parva* Muguga DNA which provided a further rationale to select it for more detailed studies. It was hoped that if the sequence detected by this probe has some homology between the two species, e.g. *T.annulata* and *T.parva*, it might mean that the gene has an important conserved functional role. Indeed sequencing of a small fragment of this probe showed homology within a cysteine proteinase gene in *T.parva* (Nene *et al*, 1990). This gene was approximately 0.8Kb in size and the authors inferred that it is probably a functionally active gene. Given these results it is likely that the gene detected by probe *TaT* 17 codes for a similar enzyme. A cysteine proteinase gene has also been isolated from *T.annulata* (Baylis *et al*, 1992). This gene was identified by using an oligonucleotide representing conserved regions of the protein. This *T.annulata* cysteine proteinase gene identified by Baylis *et al* (1992) showed low

sequence identity with the *T.parva* cysteine proteinase gene (Nene *et al*, 1990) and limited homology with the gene identified in this study suggesting that the genes belong to different classes of *Theileria* cysteine proteinase genes. However, the homology existing between the *TaT 17* gene and the *T.parva* gene (Nene *et al*, 1990) would suggest that they represent similar classes of cysteine proteinase genes. Cysteine proteinases have been shown to play an important role in the biology of some parasites. Keene *et al* (1990) showed a correlation between the cytopathic effect of virulent *Entamoeba histolytica* and the presence of a secreted cysteine proteinase which was thought to be involved in the tissue invasion. A similar role could be associated with *Theileria* cysteine proteinases but further investigations are needed to examine this hypothesis.

For the reasons listed above, probe *TaT 17* was selected to study the polymorphism between and within the Tunisian isolates. In order to define the polymorphic genotypes cloned infected cell lines were used and the results showed the presence of five different subpopulations identified by probe *TaT 17* in these clones. The five clones (1-5) used as representatives of the five subpopulations were not derived from the same parental cell line for two reasons. Firstly none of the cell lines studied included a mixture of all five genotypes; secondly it was not possible to clone every genotype present within a cell line. A better understanding of the nature of the polymorphic sites detected by probe *TaT 17* was made using DNA preparations from the five clones representative of each genotype and digested with *EcoRI* alone, *Hind III* or *EcoRI/Hind III* and probed with five fragments derived from probe *TaT 17*. These were obtained from the digestion of the *EcoRI* insert of *TaT 17* with *Hind III* (F1-F5). Each fragment was cut out, labelled and used to probe the Southern blot of the digested DNA from the five clones. The results are shown in Fig. 5.17 and the interpretation of the results illustrated in the map of each clone as presented in Fig. 5.18.

F1 was shown to be located at the right hand side of the probe as explained in the results. It detected an 8.3Kb fragment in all the *Hind III* digests (Fig. 5.17 E), which meant that the adjacent *Hind III* site outside the probe on the right side is 8.3Kb from the first one located within the fragment detected by the whole probe. That these sites are conserved and located at the same position on the genome for all clones, can be deduced since the size of the

**Figure 5.18** Restriction maps of clones 1-5 using two enzymes (*Eco*RI and *Hind* III).



**KEY:**

Sequences presenting homology

Sequences presenting homology



fragment detected in each of the clones was the same size. The polymorphism between the parasite clones was detected in double digests or *EcoRI* alone, and probed with F1. Two profiles were observed in the *EcoRI/Hind* III digests either a band of 0.7Kb or of 1.2Kb. The fact that F1 is limited by the first restriction *Hind* III site on one side and the right hand *EcoRI* site on the other side and that the fragments detected by F1 in the different clone digests are homologous, would indicate that the *EcoRI* site, on the right hand side, could be located either at 0.7Kb of the *Hind* III site in clones 1, 3 and 5 (Fig. 5.17 E, tracks 2, 8 and 14) or at 1.2Kb of the *Hind* III site in clones 2 and 4 (Fig. 5.17 E, tracks 5 and 11). This could explain the difference in *EcoRI* fragment sizes between clone 2 (7.6Kb) and clone 3 (6.8Kb) seen on the Southern blots. This size difference, of 0.8kb, is slightly different from the size difference of 0.5Kb as given by F1. This might be expected when DNA sizes are deduced from Southern blots. Clones 2 and 3, therefore, as studied by probe *TaT* 17, would only differ by the position of the right hand side *EcoRI* site.

F2 was shown to be located on the left hand side, limiting the *TaT* 17 probe. This fragment detected, on *Hind* III digests, a 4.2Kb fragment in all clones (Fig. 5.17 A, tracks 3, 6, 9, 12 and 15). This result would indicate that the two *Hind* III sites defining the 4.2Kb fragment are in the same position and conserved in all clones. Therefore the next *Hind* III site outside the locus on the left hand side is situated at 4.2Kb from the first internal *Hind* III site on the same side. The position of the 1Kb fragment, represented by F2, at the extremity of the locus, was confirmed by the fact that the 4.2Kb fragment (*Hind* III digest) was no longer detected when both *EcoRI* and *Hind* III were used, but a 1Kb fragment was observed instead. This implies that the 1Kb fragment was part of the 4.2Kb fragment and that the *EcoRI* site limiting the 1Kb fragment and the locus on the left hand side is conserved and situated at the same place in all clones. The remainder of the 4.2Kb fragment was not detected, probably because the homology between this fragment and F2 was only present in the 1Kb fragment.

Probe F2 also showed the presence of a 1.8Kb fragment in all *Hind* III digests used in *EcoRI/Hind* III digests of clones 1, 2 and 3. The first result indicates that there is a 1.8Kb fragment on the locus defined by two *Hind* III sites and showing homology with F2. When *EcoRI* is used in conjunction with *Hind* III, this fragment disappears from clones 4 and 5. This

would indicate the presence of an *Eco*RI site within this fragment in these clones. Since only one *Eco*RI site was detected in these clones (as shown previously and seen in the *Eco*RI digests), it means that the 1.8Kb fragment contains this site. Therefore the results suggest the presence of two 1.8Kb fragments in clones 4 and 5. The first corresponds to the extremity of the locus and is flanked by two *Eco*RI sites with an internal *Hind* III site situated at 1Kb from the end of the locus. The second overlaps the first fragment and starts from the same *Hind* III site, includes the internal *Eco*RI site and finishes at the next *Hind* III site on the locus situated at 1.8Kb from the first. This latter fragment is located 1Kb from the internal *Eco*RI site at 2.8Kb from the left hand side of the locus. It is necessary to postulate that a homologous sequence to F2 is present in the genome, in order to explain the presence of the 1.8Kb fragments detected. The hypothesis also explains the results obtained with the other clones. These do not contain an internal *Eco*RI site. The only 1.8Kb fragment observed is generated by the *Hind* III cuts and the use of *Eco*RI in addition to *Hind* III does not cut this fragment. The 1Kb fragment detected in clones 4 and 5 corresponds to two fragments of the same size, the first one generated for all clones from the 4.2Kb *Hind* III fragment as explained above, and the second generated from the digestion of the 1.8Kb *Hind* III fragment by *Eco*RI. These clones have the internal *Eco*RI site situated in the 1.8Kb *Hind* III fragment at 0.8Kb of the first *Hind* III site on the left hand side of the locus.

Hence the locus starts at the *Eco*RI site followed 1Kb later by a *Hind* III site, then 0.8Kb later (in clones 4 and 5) by the internal *Eco*RI site and 1Kb further on by the second *Hind* III site. Those positions are based on the distances between the two *Eco*RI sites of 1.8Kb, between the two *Hind* III sites of 1.8kb and between the end of the locus and the first *Hind* III site of 1Kb. The fact that probe F2 hybridised to the 1kb fragment obtained from the digestion of the 1.8Kb *Hind* III fragment implies that the homologous sequence between these two fragments is situated within the 1Kb fragment. Otherwise probe F2 would have hybridised to the 0.8kb fragment (generated from the 1.8Kb by *Eco*RI) if the homology was with this fragment or to both 1Kb and 0.8Kb if the homologous sequence spanned the two fragments. The results showed only the presence of the 1Kb fragment. Therefore it was postulated that the 1Kb sequence recognised by probe F2 presented a strong homology with another 1Kb

sequence situated on the same locus, the two sequences being separated by a distance of 0.8Kb.

According to the results and the hypothesis made, the five clones on the left hand side differ only by the presence, in clones 4 and 5 of an internal *EcoRI* site situated 1.8Kb from the left hand end of the locus. Otherwise they all showed the presence of two internal *Hind* III sites within the sequence of 2.8Kb at the left end of the locus. This sequence is composed of two 1Kb fragments showing high homology separated by an 0.8Kb fragment. Probe F2, was derived from *TaT* 17, which has been generated from a genomic library made from stock *TaTu* 7. This stock has only one population analogous to clone 3, when probed with *TaT* 17. Therefore, F2 could have only been generated from the 1Kb fragment limiting the locus and not from the next 1.8Kb (*Hind* III fragment), since the latter did not have the internal *EcoRI* site which is needed to generate the second homologous 1Kb fragment. This confirms the results from the ethidium bromide gel of the *EcoRI/Hind* III digests of probe *TaT* 17 which showed that F2 represented one extremity of the probe.

From the hybridisation pattern detected by F2, it could be seen that the fragment located next to F2 is identical in size to F5. The pattern detected by F5 on the same blot gives more evidence for its location next to F2. F5 detected a pattern close to that detected by F2. However the 4.2Kb and 1Kb bands were fainter and an additional 0.8Kb fragment was present in clone 4 and faintly detected in clone 5 (not visible on the photograph). This fragment was present when these clones were subjected to both restriction enzymes and its appearance indicates that F5 is following F2 on the locus. Because of the presence of the internal *EcoRI* site in clones 4 and 5, F5 would recognise on *EcoRI/Hind* III digests, a 1Kb and 0.8Kb fragments. The sum of these two fragments would represent the sequence homologous to F5 on clones 4 and 5.

F2 and F5 have some homology since F5 recognises the 1.8Kb fragment in *EcoRI* digests of all clones. When the clones are digested with both restriction enzymes a 1Kb fragment is detected. This would indicate that the homologous sequences would be represented by F2 on one hand and the 1Kb fragment contained in F5 on the other hand, located 0.8Kb from F2 (Fig. 5.18).

However the 1Kb fragment detected by F5 was faint compared to its detection by F2 (compare Fig. 5.17 A and B). This suggest that F5 has more homology with the matching sequence on the genome of the five clones than with the 1Kb sequence represented by F2. The strong signal obtained in both clone 4 and 5 could be due to the 1Kb fragment deriving from F5 and not from F2, as a result of the internal *EcoRI* site. The same explanation could be given to the fact that the 4.2Kb fragments (*Hind* III digests) were also detected by probe F5. These were recognised on the basis of the presence of the 1Kb fragment represented by F2 and showing homology with the 1Kb fragment within F5.

The two faint bands seen in clone 4, when digested with *EcoRI/Hind* III and *Hind* III, are believed to be due to poor stripping of the blot after probing with F3 and F4. in addition the quantity of DNA loaded for clone 4 was greater than the other clones, resulting in the detection of very strong bands on hybridisation and greater difficulties in stripping the blot.

Probe F3 detected the specific fragments for each clone already described when the entire probe was used on *EcoRI* digests, but it also detected a 1.4Kb fragment equivalent in size to F3 and this represents the homologous sequence of F3 in the different clones. The detection by this fragment of the 1.8Kb band, although faint in some of the clones, indicates that again there is probably a sequence with some homology to F3 on the 1.8Kb fragment obtained when the clones are cut with *Hind* III or *EcoRI/Hind* III. In addition the similar 1.8Kb fragment, recognised by F3 in *EcoRI* digests of clones 4 and 5, is not detected when the clones are digested with both enzymes. This and the detection of the 0.8Kb fragment by this probe would indicate that the homology is located in the 0.8Kb fragment of F5. This fragment is also detected faintly in clone 5, but is not visible on the photograph. Probe F3 also showed two very faint bands of 4.2Kb and 1Kb in *Hind* III and *EcoRI/Hind* III digests of clone 4. If this is not an artefact, it would suggest that the sequence with homology to F3 overlaps the *Hind* III site of F1 to a small degree so that the resulting hybridisation signal between F3 and either the entire 4.2Kb fragment or the 1Kb fragment (F2) would be very weak.

Probe F4 has a very straightforward pattern. It recognises, in addition to the specific banding for each clone detected by the whole probe *TaT* 17 (8.6-4.9Kb) in *EcoRI* digests, a

1.8Kb was only calculated from the difference in *Eco*RI digests between clone 1 and clone 3 ( $8.6-6.8 = 1.8$ ). The possibility that internal *Hind* III sites could exist within this 1.8Kb fragment, is not excluded. This would only be detected when probed with the homologous sequences from clone 1 and would be specific to this clone. The fact that the 1.8Kb fragment detected only in clone 1, is deleted from the other four clones (2-5) might mean that all clones are derived from one parental population and only differ by point mutations. This would introduce new *Eco*RI sites. An alternate explanation could be a translocation of a pre-existing *Eco*RI site.

The population corresponding in type to clone 3 would thus have derived from the one represented by clone 1 by deletion of a fragment of 1.8Kb. Clone 2 would then have been generated from clone 3 by the translocation of the external *Eco*RI site situated on F1. Clones 4 and 5 would have derived from clones 2 and 3, respectively, by point mutation and the introduction of an internal *Eco*RI site at 1.8Kb of the *Eco*RI site at the left hand side of the locus detected by probe *TaT* 17.

Two other probes, SPAG-1 (Williamson *et al*, 1989) and E2.1 (Shiels, unpublished data), were also used to detect polymorphism in the Tunisian isolates. The aim of this study is to investigate the extent of variability in *T.annulata* and since both probes showed a limited number of genomic variants in the preliminary study (two in each case) amongst the Tunisian isolates, they were not selected for further analysis. The fact that both genes were not highly polymorphic for *Eco*RI sites could be relevant in the design of a subunit molecular vaccine but was of less interest in studying the degree of heterogeneity in *T.annulata* population. However, these probes, did show that the polymorphism in the Tunisian isolates could be detected with probes generated from parasites isolated from other geographical areas.

Overall the results described in this chapter showed that DNA probes could be used to detect genomic polymorphism in *T.annulata*. The detection by these probes of the same number of fragments in piroplasm DNA and cell line DNA was an important aspect to consider for the selection of relevant probes for this study. This means that, for example, if six bands are detected in piroplasm DNA by probe *TaT* 17, they also have to be seen on Southern blots of cell line DNA. Therefore, differences observed between profiles of piroplasm and cell

line obtained from the same animal could be analysed on this basis other than the lack of consistency of the genomic probe (as illustrated by e.g. probe *TaT* 16). Accordingly, two DNA probes, *TaT* 17 and *TaT* 21, were chosen to study all the Tunisian stocks. They both give evidence for the presence of mixture of genomic variants, within the isolates, which could be separated by cloning *T.annulata* infected cell lines. These probes together with MAb 7E7 and GPI isoenzyme electrophoresis are used in the next chapter to characterise the *T.annulata* parasite population from Tunisia.

## ***CHAPTER SIX***

### ***POLYMORPHISM IN THEILERIA ANNULATA ISOLATES***



## 6.1 INTRODUCTION

The aim of this study was to assess the extent of diversity in *Theileria annulata* isolates from a single country. Three approaches were used in the characterisation of the parasite. In the first, antigenic diversity was examined based on the variable reactivity of an anti-schizont MAb. In the second, isoenzyme variation was examined using the difference in migration of the glucose phosphate isomerase enzyme in an electric field by electrophoresis. In the third, variation in the restriction enzyme site polymorphism was examined using a series of gene probes that detected simple, reproducible banding patterns that differed for many of the isolates.

To date most studies of variation in *T.annulata* have compared isolates from widely different geographical regions and little information is available on the extend of parasite variation within a defined geographical region and, in particular, it is not known whether particular variants are localised to specific areas within a region. This study is aimed at defining such levels of variation and therefore investigating, at a fundamental level, how important a factor parasite variation may be in the design of effective control measures. The diversity in *T.annulata* parasites is described using a combination of MAb reactivity, isoenzyme analysis and DNA probes. As a consequence, the study has extended the previous level of knowledge of diversity in *T.annulata*.

Studies, *in vivo*, have shown that different field strains of *T.annulata* exhibit different levels of virulence in cattle (Pipano *et al*, 1974). A better understanding of virulence and protection might be provided by study of genotypically and phenotypically well defined stocks which could also be used for cross-immunity trials and provide potential candidates for vaccination.

At the present time it is not known which molecules are responsible for protection and therefore it is difficult to predict how cross-protective a given vaccine strain is likely to be, except by direct experimentation. Variation both within strains and between strains of *T.annulata* has been shown for a number of phenotypes and genotypes by studies on merozoite production *in vitro* (Shiels *et al*, 1992), monoclonal antibody profiles (Shiels *et al*, 1986a), isoenzyme profiles (Melrose *et al*, 1984), a sporozoite surface antigen gene



(Williamson *et al*, 1989), and merozoite/piroplasm antigens (Dickson and Shiels, 1993). Even though these studies have been dealing with a limited number (1-12) of isolates, it is clear from these studies that parasite variation occurs and may be an important factor in the development of any subunit vaccine as well as in the production of vaccine strains for use in specific geographical regions.

## 6.2 POLYMORPHISM OF TUNISIAN *T.ANNULATA*

### 6.2.1 *Isolates from a single country.*

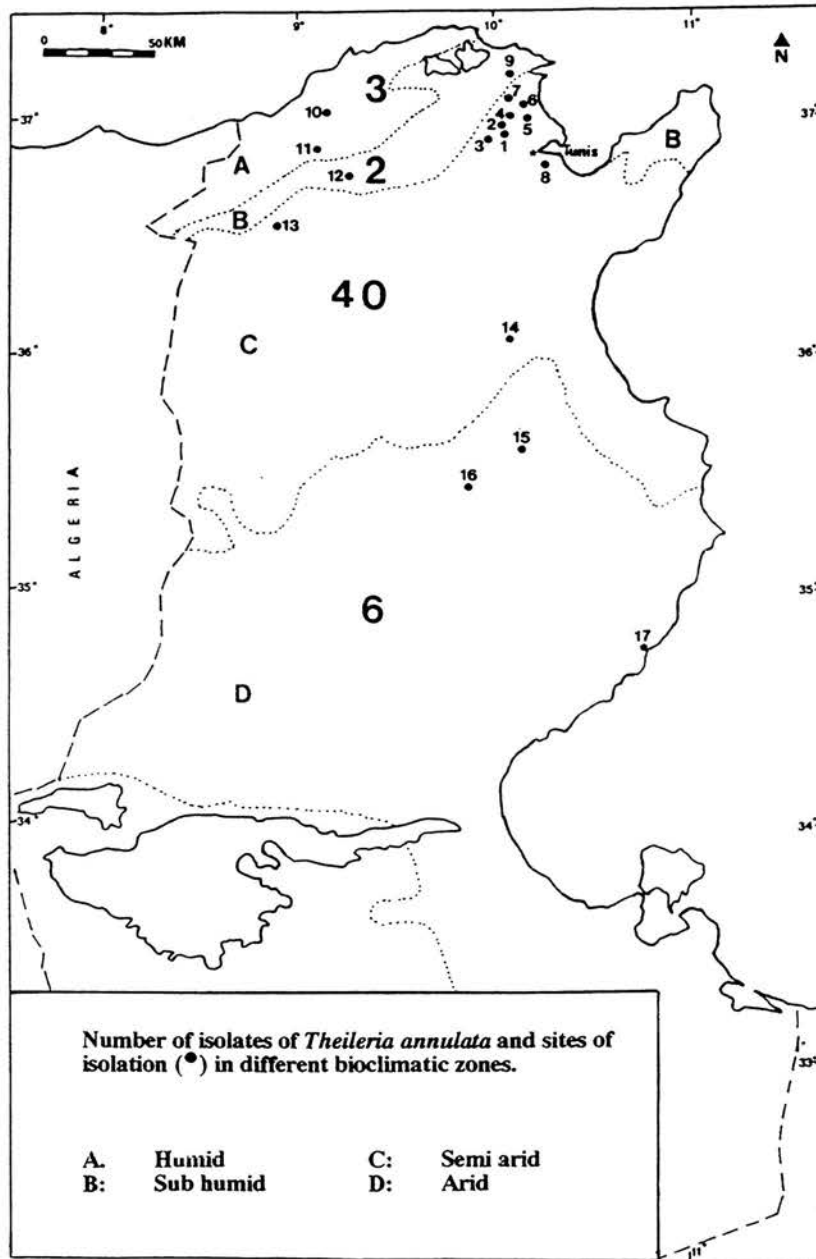
The samples were collected from 17 different sites of isolation in four bioclimatic zones within Tunisia (Fig. 6.1). From these samples, 51 *T.annulata* stocks have been isolated and have been characterised using the three different methods of analysis described in the previous three chapters. The results of this analysis with all isolates is given in Table 6.1. The isolates comprised schizont-infected cell lines established *in vitro* (48 cell lines were studied) and piroplasm preparations purified from infected erythrocytes collected from animals (12 piroplasm isolates were analysed).

#### 6.2.1.1 Antigenic polymorphism.

The preliminary study at the antigenic level showed that two techniques, IFAT and Western blotting, could be used to detect variability in *T.annulata* stocks. Ten MAbs were selected on the basis of their pattern of IFAT reactivity with schizonts from the cell line used to immunise the mouse (*TaTu* 1/270) and their lack of reactivity with uninfected bovine PBM. These MAbs were then tested on a further nine schizont-infected cell lines. Only MAb 7E7 displayed a variable reactivity against these stocks. Indeed MAb 7E7 stained 1-2% of the schizonts in two stocks, reacted with all schizonts in four stocks and showed no reaction against three stocks. This initial investigation suggested that MAb 7E7 detected antigenic diversity in *T.annulata* stocks and was therefore used to screen the remaining 48 schizont-infected cell lines by IFAT. (These cell lines were also tested with the other nine MAbs to confirm the lack of variable reactivity of the latter). Variation in the percentage of schizonts recognised by MAb 7E7 in the infected cell lines, was observed

**Figure 6.1** Map of Tunisia showing number of isolates of *T.annulata* (large numbers) and sites of isolation (small numbers) in different bioclimatic zones.

# TUNISIA



**Table 6.1** Comparison of *T.annulata* infected cell lines isolated from different bioclimatic zones.

TaTu stocks	Bioclimatic zone	Site of isolation	Parasite material used	MAB 7E7 reactivity	GPI types	TaTu 17 profiles	TaTu 21 profiles
37	A	10	CL	100%	1+4	1+2+4	1+2
38		11		100%	4+6	3+4+5	1+3
39		11		100%	2+4	5	1
35	B	9	CL	0%	6	3	1
36		12		15%	2+4+7	3+5	1
7	C	3	P	-	4	3	1+3
8		3	P	-	ND	2+3	1
9A		3	CL	100%	2+4	(2)+3+4+5	1+2
			P	-	ND	(2)+3+4+5	1+2
			Clone 4	100%	2	4	1
9B		3	CL	100%	2+4	2+3+4+5	1+3
			P	-	ND	2+3+3+5	1+2+3
			Clone 2	100%	4	2	1
			Clone 3	100%	2	3	1
10		3	P	-	ND	2	1
11		2	CL	100%	1+2+4+6	3	1
			P	-	2	3+4	1
12		3	CL	100%	4+6	3+5	1
			P	-	4	3+5	1
13		3	CL	100%	6	1	2
			P	-	4	1	2
			Clone 1	100%	6	1	2
14A		1	CL	0%	4+6	3+(4)	2
14B		1	CL	0%	4	5	1
			P	-	ND	(3)+5	1
14C		1	CL	15%	4+6	4+5	1
			P	-	2+4	3+5	1+2
15		4	CL	40%	4+6	2+3	3
16		4	CL	100%	3+6	2	2
17		5	CL	0%	2+4	3	3
18A		4	CL	100%	1+5	3+4	3
			P	-	1+2+4	3+4+5	1+2+3
18B		4	CL	100%	4	3+5	2
			P	-	(1)+2+4+6	3+(5)	1+2+3
18C		4	CL	100%	2+4+6	3	3
19		4	CL	100%	4	3	3
20		4	CL	100%	2+4+6	3	1
21		4	CL	100%	2+4+6	5	1
23		4	CL	100%	2+4	2	1+3
24A		3	CL	1%	6	3	2
24B		3	CL	25%	2+4+6	(2)+3+5	1+2
25		6	CL	100%	4	2	1+3
26		6	CL	100%	2	ND	ND
27		6	CL	100%	2+6	2+3	2
28		6	CL	50%	2+4	3	1
29		6	CL	100%	6	2+4	1+3
30		6	CL	100%	2+4	3+5	1+2+3
31		6	CL	15%	4+6	3+4	1
32		6	CL	40%	2+4	3+5	1+2
33		7	CL	100%	2+4+6+7	2+3+5	1
34		8	CL	30%	4	3+5	1
40		13	CL	20%	1+4+7	3+5	1+2
41		13	CL	20%	2+4	3+5	1
42		13	CL	0%	2	2	1
43		13	CL	10%	2+5	2+3	1
44		13	CL	100%	1+4	2	1
45		13	CL	50%	2+4	2+3+5	1
48		14	CL	5%	2	1+3	1
49	D	15	CL	40%	4	2+3+4+5	1+2+3
46		16		100%	4+6	4	1
47		16		30%	4	2+3	1
50		16		50%	2+4	3+5	1+2
51		17		100%	2+4+6	3+5	1+(2)+(3)
52		17		100%	4	2+3	3

( ) = Faint bands

CL = cell line

P = Piroplasms

ND = not done

with the proportion of positive cells ranging from 0%-100% (Fig. 6.2). When cell lines showing intermediate percentages of staining were cloned by limiting dilution, the cloned cell lines segregated into either totally negative cell lines (0% positive cells: 7E7-) or totally positive cell lines (100% positive cells: 7E7+). The cell lines in which only a proportion of the schizonts were positive can be interpreted as consisting of a mixture of the two phenotypes 7E7+ and 7E7-.

The stage specificity of the expression of the antigen recognised by this monoclonal was determined by demonstrating that the MAb reactivity with intra-erythrocytic piroplasms was always negative even when cell line from the same isolate was 100% positive. This suggests that the antigen is stage specifically expressed in the schizont.

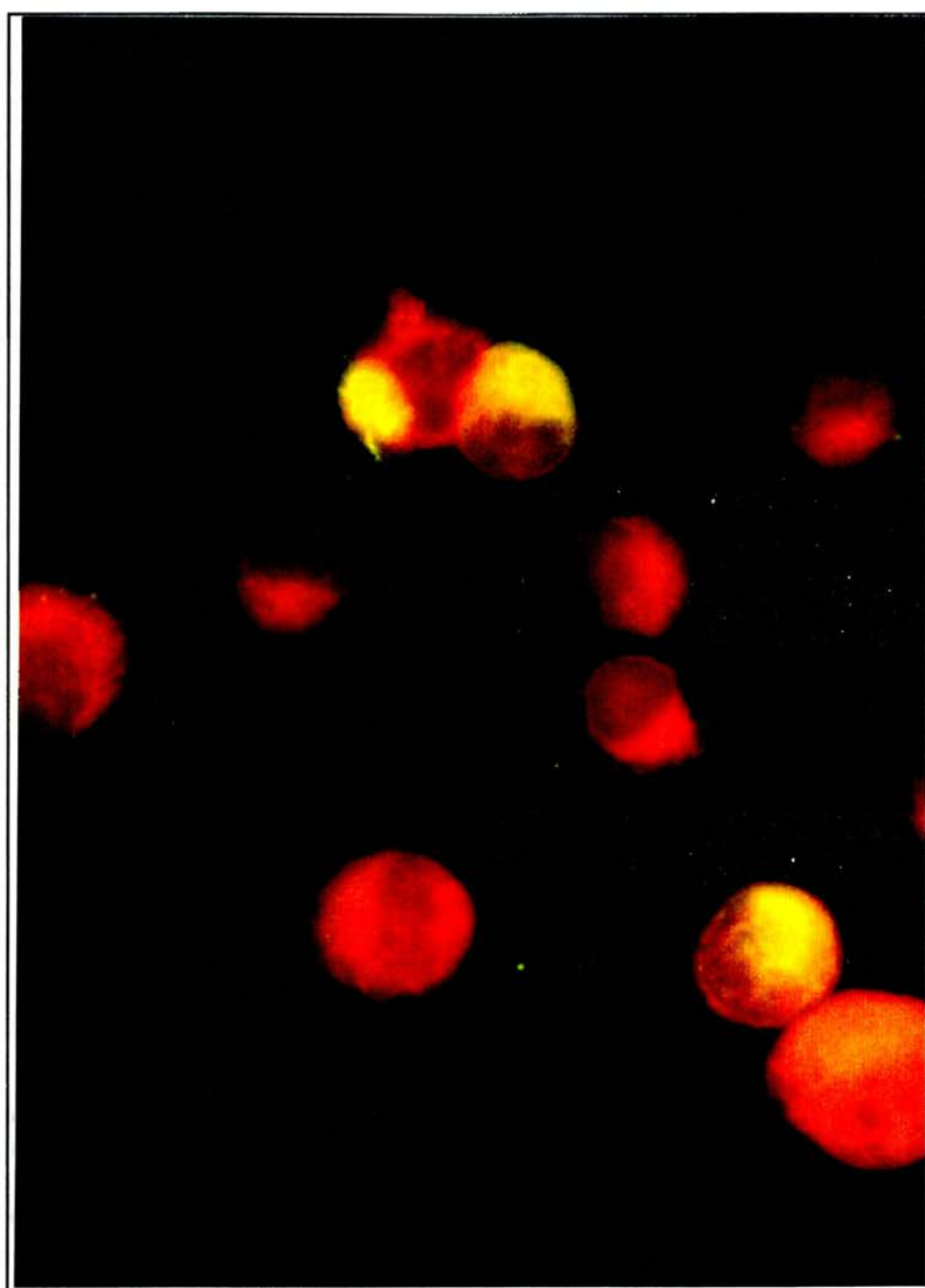
The 10 MAbs were screened by Western blotting of extracts of different parasite stocks, of these MAbs 3D10 and 19E11 detected the same antigen which varied in size between isolates (130/148kDa) while MAbs 12D5 and 17H2 detected the same antigen which also varied in size between isolates (175/180kDa). Due to technical difficulties in obtaining clear cut reproducible results using schizont extracts, these MAbs were not used further in this study. MAb 7E7 detected a 38kDa protein in extracts from schizont infected cell lines showing a positive reactivity with this MAb by IFAT. Since the Western blotting did not detect more variation using MAb 7E7 than IFAT, it was not used to study all the schizont infected cell lines.

#### **6.2.1.2 Enzymatic polymorphism.**

When the 51 isolates were examined for their GPI phenotype the majority showed a multiple triplet band pattern. Seven variant forms of GPI triplets were identified. They were classified according to the extent of their migration from the most anodal (1) to the most cathodal (7). On the basis of the three banded patterns given by individual clones, each multiple banded type has been scored accordingly to its composite GPI pattern.

#### **6.2.1.3 Genomic polymorphism.**

**Figure 6.2**    Reactivity of MAb 7E7 using IFAT on schizont-infected cell line *TaTu* 49 (40% 7E7<sup>+</sup>).



Polymorphism at the level of the genome within and between isolates was examined using two polymorphic gene probes (*TaT* 17 and *TaT* 21). Probe *TaT* 17 identified five different genotypes present either as a single profile or as a mixture of profiles of different variants (Fig. 6.3). The five profiles obtained using DNA from cloned schizont-infected cell lines, respectively clones 1 to 5, probed with *TaT* 17, suggest that the gene fragment is single copy in the genome and varies in both size and the number of *Eco*R1 sites it contains. No internal *Eco*R1 site was detected in probe *TaT* 17 which was derived from the *T.annulata* stock *TaTu* 7 used to generate the genomic library. DNA from this stock showed a single *Eco*R1 fragment (6.8Kb) (see Fig. 5.4, track 2), a result consistent with the lack of an internal *Eco*R1 site. When DNA from clones 1, 2 and 3 was probed with *TaT* 17, each showed a unique fragment of 8.6Kb, 7.6Kb and 6.8Kb respectively. DNA from clones 4 and 5 showed bands of 5.6Kb and 4.9Kb respectively, and an additional 1.8Kb fragment in both, which indicated that the locus detected by this probe contains an additional *Eco*R1 site spanned by probe *TaT* 17 (see Fig. 5.6).

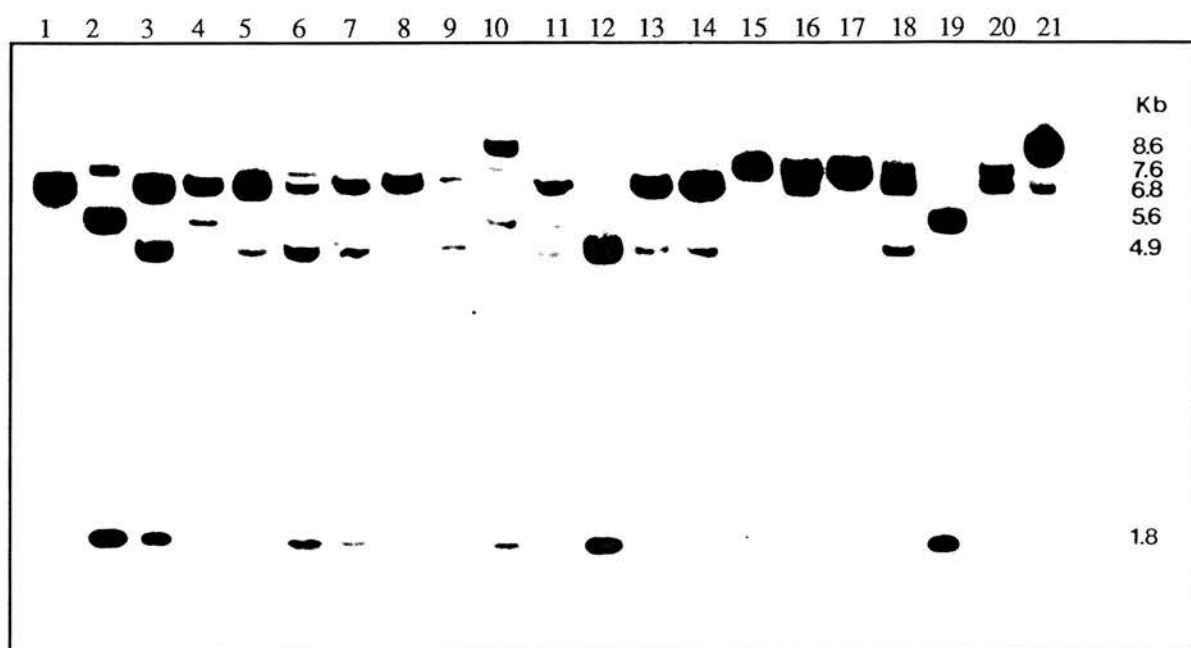
Profiles, composed of three basic patterns of a 7.7Kb fragment (profile 1), a 4.3Kb and 3.9Kb fragments (profile 2) and a 4.5 and 3.7Kb fragments (profile 3) were detected by probe *TaT* 21.

#### 6.2.1.4 Analysis of the polymorphism using the above markers.

The frequency of the different variants as defined by GPI profiles and the profiles obtained with the *TaT* 17 probe were analysed using the data from the schizont-infected cell lines. One of the cell lines, *TaTu* 26, was only studied by GPI and MAb 7E7, because the stablate proved to be inviable, and so only the remaining 47 cell lines were studied (Table 6.1); additionally the data from homologous piroplasms of certain stocks was not included. The frequency of the different GPI types is illustrated in Table 6.2, together with the proportion of stocks with different numbers of variants. The most common were variants 4, 2 and 6 and these were also the most common variants when only homogenous stocks were analysed (Table 6.4). Similarly these variants were the most common



**Figure 6.3** Southern blot of 21 Tunisian *T.annulata*-infected cell lines digested with *Eco*RI and hybridised with probe *TaT* 17.



**Table 6.2** Distribution of the different GPI variants within the 47 *T.annulata* infected cell lines and their mixtures.

GPI variants	1	2	3	4	5	6	7
Number of stocks including these variants	5	23	1	36	2	20	3
Frequency	11%	48%	2%	77%	4%	42%	6%
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Number of variants within the stocks			1V	2V	3V	4V	
Number of stocks			14	24	7	2	
Frequency			30%	51%	15%	4%	
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**Table 6.3** Distribution of the different genomic profiles defined by probe *TaT* 17 within the 47 cell lines and their mixtures.

<i>TaT</i> 17 variants	1	2	3	4	5
Number of stocks including these variants	3	19	34	11	20
Frequency	6%	40%	72%	23%	43%
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Number of variants within the stocks		1V	2V	3V	4V
Number of stocks		18	21	5	3
Frequency		38%	45%	11%	6%
<hr/>					

V = Variant

**Table 6.4**      Frequency of the different GPI variants within the isolates with regards to each specific variants.

Single variants	1	2	3	4	5	6	7
Number of stocks (14T)	0	2	0	8	0	4	0
Frequency		14%		58%		29%	
Mixture of two variants	2/4	4/6	1/4	1/5	2/5	2/6	3/6
Number of stocks (24T)	11	7	1	1	1	1	1
Frequency	48%	30%			22%		
Mixture of three variants			2/4/6	2/4/7	1/4/7		
Number of stocks (7T)			5	1	1		
Frequency			71%	29%			
Mixture of four variants			1/2/4/6	2/4/6/7			
Number of stocks (2T)			1	1			
Frequency			50%	50%			

T: Total

components of mixed stocks where the most abundant class consists of a mixture of two types (Table 6.2).

The distribution of *TaT* 17 variants in the population was not equal with variants 3, 2 and 5 being the most frequent and variant 4 being detected in the almost a quarter of the isolates (Table 6.3). Variants 3, 2 and 5 were also the most frequent in the mixtures and in the cell lines that only showed a single component (Table 6.5). Again the most frequent mixture seemed to be represented by two profiles (Table 6.3), followed by stocks showing only one variant. This was also true by GPI analysis (Table 6.2). However, when the profiles of GPI and probe *TaT* 17 are considered together, it was found that many of the stocks which were homogenous for one marker were mixtures when defined by the other marker. On this basis only half of the initial number of stocks identified as homogeneous were homogeneous for both markers. Therefore it is very difficult to assume that a stock is composed of only one homogenous population equivalent to the clones obtained *in vitro*. As only four markers have been used, the results will only give a strong probability that a given stock is "clonal". In this study, using the four markers together, five out of the 47 cell lines could be classified as potentially clonal. These are stocks *TaTu* 35, 13, 14B, 19 and 42 (Table 6.1).

In conclusion from the study of the established infected cell lines, the frequency of different parasite genotypes/phenotypes is variable and mixtures are more frequent than homogeneous populations. Additionally, if the four markers are to be considered together there is a very high level of polymorphism in the *T.annulata* parasite populations collected from a single country (Table 6.1). In the next section, the observed polymorphism is considered in relation to the geographical bioclimatic zones of isolation.

### **6.2.2 Isolates from different bioclimatic zones.**

When zones A, B, C and D (Fig. 6.1, Table 6.1) were compared it can be seen that the majority of the isolates were mixtures. The complete range of seven GPI variants was only found in zone C, including variant 3 which was detected once in cell line *TaTu* 16, isolated from site 5. Isolates from zone A, were homogenous for the 7E7+ phenotype which

**Table 6.5** Frequency of the different genomic variants, defined by probe *TaT* 17, within the isolates with regards to each specific variant.

Single variants	1	2	3	4	5	
Number of stocks (18T)	1	5	8	1	3	
Frequency	5.5%	28%	44%	5.5%	17%	
Mixture of two variants	3/5	2/3	3/4	1/3	2/4	4/5
Number of stocks (21T)	9	6	3	1	1	1
Frequency	43%	29%	14%		14%	
Mixture of three variants		2/3/5	1/2/4	3/4/5		
Number of stocks (5T)		3	1	1		
Frequency		60%	20%	20%		
Mixture of four variants			2/3/4/5			
Number of stocks (3T)			3			
Frequency			100%			

T: Total

was relatively rare in zone B but occurs at a relatively high frequency in zone D. However in zone C all possible frequencies of the 7E7+ phenotype were observed, ranging from 0-100%. This zone, because of the large number of stocks analysed may reflect a more accurate sample of the real distribution of each marker within a given region. The apparent absence of certain variants within particular zones could be partly due to the low frequency of these variants in the total parasite population. Overall the polymorphism detected in the four different zones has a similar pattern of variation and would indicate that the distribution of the different type of variants is not correlated with specific bioclimatic zones.

#### ***6.2.3 Isolates from different sites of isolation in one zone.***

Several isolates from the same sites were made in zone C allowing the analysis of the level and nature of polymorphism between sites (Table 6.6). None of the sites appeared homogeneous for any of the markers studied. In fact, similar levels of variation were observed when the different sites were compared with each other. In addition, the level of variation observed within a site was similar to the level of variation observed within a zone. Overall the data indicate that there is no correlation between site of isolation and specific type of variants.

#### ***6.2.4 Isolates from different farms on one site.***

The different stocks presented in Table 6.6 from the same site were collected from different farms (each stock number correspond to one farm). The results from analysis of these stocks show that there is no correlation between the variants and specific farms at the same site of isolation e.g. GPI variant 2 was isolated from more than one farm on site 4.

#### ***6.2.5 Isolates from different animals on same farms.***

The characterisation of the cell lines collected from different animals on single farms are presented in Table 6.7. Some of the farms are homogeneous for the 7E7+ phenotype, but in general they show a range of variation in the other markers studied.

**Table 6.6** Comparison of *T.annulata* infected cell lines isolated from the same sites in zone C

TaTu stocks	Site of Isolation	MAb 7E7 reactivity	GPI types	TaT 17 profiles	TaT 21 profiles
15	4	40%	4 + 6	2 + 3	3
16		100%	3 + 6	2	2
18A		100%	1 + 5	3 + 4	3
18B		100%	4	3 + 5	2
18C		100%	2 + 4 + 6	3	1
19		100%	4	3	3
20		100%	2 + 4 + 6	3	1
21		100%	2 + 4 + 6	3 + 5	1
22		100%	4	5	1
23		100%	2 + 4	2	1 + 3
25	6	100%	4	2	1 + 3
27		100%	2 + 6	2 + 3	2
28		50%	2 + 4	3	1
29		100%	6	2 + 4	1 + 3
30		100%	2 + 4	3 + 5	1 + 2 + 3
31		15%	4 + 6	3 + 4	1
32		40%	2 + 4	3 + 5	1 + 2
40	13	20%	1 + 4 + 7	3 + 5	1 + 2
41		20%	2 + 4	3 + 5	1
42		0%	2	2	1
43		10%	2 + 5	2 + 3	1
44		100%	1 + 4	2	1
45		50%	2 + 4	2 + 3 + 5	1
9A	3	100%	2 + 4	(2) + 3 + 4 + 5	1 + 2
9B		100%	2 + 4	(2) + 3 + 4 + 5	1 + 3
12		100%	4 + 6	3 + 5	1
13		100%	6	1	2
24A		1%	6	3	2
24B		25%	2 + 4 + 6	(2) + 3 + 5	1 + 2

( ) Faint bands



**Table 6.7** Comparison of *T.annulata* infected cell lines isolated from animals on the same farm in zone C.

<i>TaTu</i> stocks		Site of isolation	MAb 7E7 reactivity	GPI types	<i>TaT17</i> profiles	<i>TaT21</i> profiles
9	A	3	100%	2 + 4	(2) + 3 + 4 + 5	1 + 2
	B		100%	2 + 4	2 + 3 + 4 + 5	1 + 3
14	A	1	0%	4 + 6	3 + (4)	2
	B		0%	4	5	1
	C		15%	4 + 6	4 + 5	1
18	A	4	100%	1 + 5	3 + 4	3
	B		100%	4	3 + 5	2
	C		100%	2 + 4 + 6	3	1
24	A	3	1%	6	3	2
	B		25%	2 + 4 + 6	(2) + 3 + 5	1 + 2

( ) Faint bands

Thus the heterogeneity of *T.annulata* parasites extended even to animals on the same farm and again a similar level of variation is detected across regions as detected in single farms.

#### **6.2.6 Isolates from the invertebrate tick host and the vertebrate bovine host on the same farm.**

Two batches of ticks were collected from two farms, Bechir and Chergui (site of isolation 23), in the highly endemic area of Bouhanach. One animal suffered from clinical theileriosis at the Chergui farm and one cell line was isolated from its PBM, cell line *TaTu* 23. The two batches of ticks were used to infect naive PBM *in vitro* and the cell lines obtained were studied by GPI analysis. They both showed the same profile which was different from the profile shown in GPI by cell line *TaTu* 23 (data not shown). Lysates for GPI analysis were also prepared from the salivary glands of the Chergui ticks. The GPI profiles showed by the salivary gland lysate and *TaTu* 23 cell line lysate were identical (data not shown).

From these data it could be assumed that the cell line set up *in vitro* from the Chergui ticks was contaminated during the *in vitro* manipulation by the Bechir ticks explaining the similar profiles detected in the two cell lines established from the ticks *in vitro*. Another eventuality is the mixing of the two batches of ticks during transport from Tunisia to Scotland.

The GPI analysis of the Chergui tick salivary glands was performed in Tunisia. The fact that both salivary gland lysate and *TaTu* 23 cell line lysate presented identical profiles when analysed by GPI electrophoresis would indicate that the same population of parasite is circulating amongst the invertebrate and vertebrate hosts of *T.annulata* in this particular farm.

#### **6.2.7 Conclusion.**

The variation analysed in this study showed that there is a high level of polymorphism in *T.annulata* isolates and that there is no characteristic common to stocks

from one country, nor to stocks isolated from one bioclimatic zone. Similarly extensive polymorphism is also found in one zone between isolates within a site and within a single farm showing that the diversity extended to parasite stocks collected from one site of isolation and even a single farm. This would suggest that the populations of parasites are panmictic across the whole of Tunisia with no obvious level of population isolation within the country. However on one occasion, no diversity was detected between the parasite isolated from the ticks and the cattle living on the same farm. This result is based on only one analysis and more data are needed to assess the significance and reliability of such a finding.

### **6.3 ASSESSMENT OF THE RELIABILITY OF THE METHODS USED TO INVESTIGATE THE POLYMORPHISM**

The material used for this study was mainly derived from schizont-infected cell lines. This raises the question as to whether such a system provides an accurate representation of the parasite variants within the individual animals sampled. Three approaches were undertaken to examine this.

Firstly, a comparison was made between the GPI and DNA probe profiles of piroplasms and schizont-infected cell lines, isolated from the same animal at the same time. The results of this analysis are presented in Table 6.8 for isolates from Zone C. It is clear that the variants defined could substantially differ depending on whether schizonts or piroplasms were examined. In three of the samples (14C, 18A and 18C) a greater number of variants were detected in the piroplasms from the same animal, suggesting that some type of selection reducing diversity may be occurring in culture. However the data from isolate *TaTu* 11 does not support this, as the cell line showed greater heterogeneity than the piroplasm preparation. Clearly, the method of isolating the parasite is important and requires further investigation, although if the selection observed was random with respect to the different variants it should not affect the analysis.

Secondly, and to examine this question further, three calves were infected experimentally, each with a cell line containing a mixed parasite population, piroplasms

**Table 6.8** Comparison of *T.annulata* macroschizont infected cell lines (CL) with their homologous prioplasms (P)

<i>T.annulata</i> stocks	Parasite material	GPI types	<i>Ta</i> T17 profiles	<i>Ta</i> T21 profiles
11	CL	1 + 2 + 4 + 6	3	1
	P	2	3 + (4)	1
13	CL	6	1	2
	P	4	1	2
14C	CL	4 + 6	4 + 5	1
	P	2 + 4	3 + 5	1 + 2
18A	CL	1 + 5	3 + 4	3
	P	1 + 2 + 4	3 + 4 + 5	1 + 2 + 3
18C	CL	2 + 4 + 6	3	1
		(1) + 2 + 4 + 6	3 + (5)	1 + 2 + 3

( ) Faint bands

and cell lines were subsequently obtained from them and typed as described previously. The results showed that overall the number and identity of variants recovered in piroplasms differed to those found in the cultured cell lines (compare CL with P profiles, Table 6.9). The differences between the profiles of the cell lines and the profiles of the piroplasms (derived from these calves) were similar to those found when the same comparison is made using naturally infected animals (Table 6.8) as described above. In addition, not all the variants present in the cell line used for infection were recovered from the infected calf in either the piroplasms or the cell lines.

To further investigate the possibility of selective sampling, calf 400 was infected with cell line *TaTu* 11 and *H.a.excavatum* ticks were fed on it, in order to pick up circulating piroplasms (Fig. 6.4). Both infected cell line and piroplasms were obtained from the calf; sporozoites from the ticks were used to infect *in vitro* PBM collected from a naive calf (calf 260). The derived cell line *TaTu* 11/400/260 was used to make comparisons, using GPI analysis, with the *TaTu* 11/400 cell line and piroplasms, (derived from calf 400) and the original cell line and piroplasms stock *TaTu* 11 derived from the field isolate 11. The results are shown in Fig. 6.5. The GPI type of piroplasms stock 11/400 has suffered from denaturation of the proteins during transport from Tunisia to Scotland. This sample had been checked before it was transported and only variant 4 was present. Fig. 6.5 shows the original cell line (Tracks 2 and 8) and piroplasms (Track 3) from stock *TaTu* 11; the cell line established from calf 400 (Track 4), the lysate obtained from the salivary glands of the ticks fed on calf 400 (sporozoites) (Track 5); the piroplasms collected from calf 400 (Tracks 1, 6 and 9) and the cell line established from infection *in vitro* of PBM 260 with the tick fed on calf 400 (Track 7).

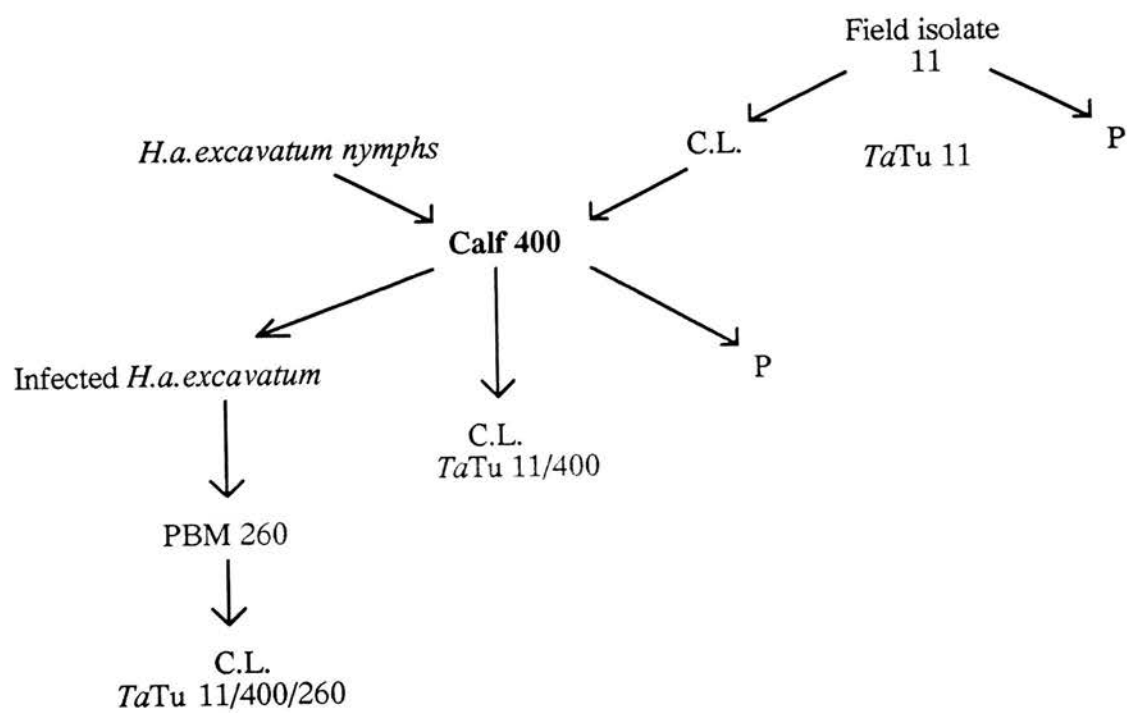
As seen in Fig. 6.5, the cell line *TaTu* 11/400/260 obtained by infection *in vitro* from tick salivary glands (Track 7) showed similar profile to the salivary gland themselves (Track 5). The cell line obtained from calf 400, although variant 4 is not easily visible on the photograph was similar, with regards to the number and type of variants present, to the cell line established from the ticks (Tracks 4 and 7). Therefore three variants were transmitted to calf 400 from cell line *TaTu* 11 and have also been producing piroplasms but

**Table 6.9**      Comparison of *T.annulata* stocks isolated from experimentally infected calves (\*) with their parental cell lines (•)

<i>TaTu</i> stocks	Parasite material	MAB 7E7 reactivity	GPI types	<i>TaT17</i> profiles	<i>TaT21</i> profiles
• 2	CL	1%	1 + 4	3 + 5	1 + (2)
* 2/029	CL	0%	4	5	1
	P	-	4	3 + 5	1
* 2/164	CL	0%	4	5	1
• 9A	CL	100%	2 + 4	(2) + 3 + 4 + 5	1 + 2
* 9A/170	CL	100%	4	(3) + 5	1
	P	-	4	3 + 5	1

CL      Cell line  
P      Piroplasms  
( )      Faint bands

**Fig. 6.4**      Diagram of origin of stocks derived from *TaTu* 11

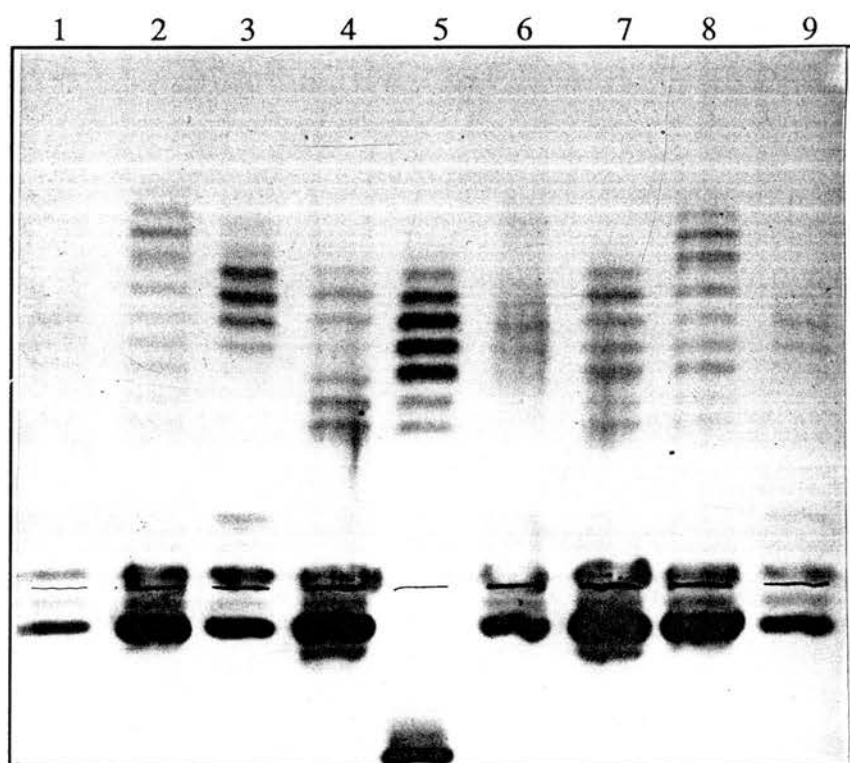


C.L.: Cell Line  
P: Piroplasms

**Figure 6.5** GPI isoenzyme patterns of *T.annulata*. Tracks correspond to stocks.

Tracks	<i>T.annulata</i>
1, 6 and 9	<i>Ta</i> Tu 11/400 piroplasms
2 and 8	<i>Ta</i> Tu 11 cell line
3	<i>Ta</i> Tu 11 piroplasms
4	<i>Ta</i> Tu 11/400 cell line
5	<i>Ta</i> Tu 11/400 sporozoites
7	<i>Ta</i> Tu 11/400/260 cell line





at a level where only the ticks, by concentrating the blood, could show their presence. Indeed variant 2 and 6 were not detected on the piroplasm preparations from calf 400. A similar explanation could be then suggested to the absence of variants 1, 3 and 4 from the piroplasm preparation of stock *TaTu* 11.

This experiment has shown again that not all the variants are transmittable *in vivo*. Since variant 1 was detected neither in the cell line established from calf 400 nor in the tick salivary gland lysate. Variant 1 was also lost in the two previously infected calves using a different cell line, *TaTu* 2. Indeed whether schizont infected cell line or piroplasms were analysed no traces of this variant was found (Table 6.9). This might suggest that this particular type is inconsistently transmitted during experimental infection *in vivo* using cell lines.

Variant 4 which was clearly dominant in the tick salivary gland preparation was the only detectable variant in the piroplasms but was not predominant in the cell line. This result could be explained, if this variant underwent differentiation better than the two others, and so produced a relatively larger amount of piroplasms; additionally, since the level of differentiation is high, this particular variant would not be highly represented in the circulating mononuclear recipient cells used to establish the cell line (*TaTu* 11/400).

Overall these experiments, carried out *in vivo* and *in vitro*, showed that not all the variants have the same ability to be transmitted *in vivo*, nor to be recovered from infection *in vivo* using cell lines. The latter might affect the frequencies calculated for each variant, although from calf 170 infected with cell line *TaTu* 9A and calf 400 infected with cell line *TaTu* 11, apparently all the populations established in the animals were recovered in the cell lines derived from them. Hence schizont-infected cell lines established *in vitro* from PBM of infected animals undergoing clinical theileriosis are a reasonable sample for assessing the extent of polymorphism in the parasite population of a given country, but one should bear in mind that the number of recovered variants from a particular animal might be underestimated. This might affect, to a minor extent, the frequency of the different variants calculated above. To investigate the situation in the field, comparison between infected ticks collected from the wall of a farm where a theileriosis case had subsequently

occurred and the cell line recovered from the sick animal was undertaken using GPI analysis. Two batches of ticks were collected from two different farms but the disease was noticed in only one of them, farm 23. The tick salivary gland lysate and the cell line produced the same profile by GPI, the two variants present in the cell line *TaTu* 23, 2 and 4, were also the only ones present in the tick salivary gland lysate. This GPI analysis was undertaken in the ENMV (Tunis) where, at that time, there was no photography facilities to illustrate this finding.

Thus the sampling method used was certainly not dramatically inaccurate and had provided a large number of laboratory stocks in their original host cells which could be used for further studies.

#### 6.4 DISCUSSION AND CONCLUSIONS

The aim of this study, overall and within this chapter, was to monitor the extent of heterogeneity of *T.annulata* in Tunisia. The data presented shows that considerable polymorphism exists amongst the parasite population within this single country. As the genome of *T.parva* is known to be haploid (Morzaria *et al*, 1992a), it is assumed that *T.annulata* is also haploid. Therefore the variation seen in these stocks is almost certainly due to the presence of mixed parasite genotypes which could be separated by limiting dilution cloning. Up to four variants within one parasite stock were detected by either GPI electrophoresis, e.g. *TaTu* 11 or genomic analysis e.g. *TaTu* 9A (Table 6.1). Using all four markers no two isolates had the same profile, even when isolates from animals on the same farm were examined (Table 6.7), nor was there any correlation between the marker profiles of isolates from the same site (Table 6.6). Previous studies have shown variation between *T.annulata* populations from different countries using MAbs (Shiels *et al*, 1986a). The presence of mixed parasite populations have also been reported for *T.annulata* (Shiels *et al*, 1986a; Williamson *et al*, 1989) and for *T.parva* (Toye *et al*, 1991). Polymorphism in *T.parva* stocks was detected using phenotypic (Pinder and Hewett, 1980; Minami *et al*, 1983; Conrad *et al*, 1987b) and genotypic analyses (Conrad *et al*, 1987a, 1989; Allsopp and Allsopp, 1988). The studies on *T.annulata* mentioned only analysed a limited number of

isolates (3-12), however the present work has examined *T.annulata* parasite populations using a large number of isolates (51) from a single country and the analysis was carried out by both phenotypic and genotypic markers.

Diversity was detected between the isolates and between different samples taken from parasite stages from the same animal; for example between schizont-infected cell lines and their derived piroplasms. In general more variants were detected in piroplasms than in schizont-infected cell lines using genomic probes. This could be explained by the selection of parasite populations during the establishment of the cell lines *in vitro*. However from GPI analyses with some isolates the opposite was shown. This was well illustrated by the isolate *TaTu* 11 where four GPI types were detected in the schizont-infected cell line but only one in the piroplasms (Table 6.8).

It has been found that cloned schizont-infected cell lines have differing abilities to differentiate to the merozoite stage *in vitro* (Shiels *et al*, 1992). The subpopulations which are not present in the piroplasm preparation might be those which have a diminished ability to differentiate and infect erythrocytes, producing a low level of piroplasms of this type. Alternatively selection for minor populations which are not observed in the piroplasm sample could be occurring in the generation of the cell line. This hypothesis is directly supported by the experimental infection of calf 400 with cell line *TaTu* 11 as shown above (Section 6.3).

From experimental infection of calves with schizont-infected cell lines, it was noticed that changes occurred in both the relative proportions and the type of variants recovered from the infected animals (Table 6.9). Calves 029 and 164 were infected with cell line *TaTu* 2 in which two variants were detected by probe *TaT* 17. Only one variant was present in the cell lines isolated from these calves as assessed by the same probe. However the analysis of the piroplasms DNA from *TaTu* 2/029 with probe *TaT* 17 showed the presence of both genotypes (Table 6.9). This could suggest that particular variants grow better in the bovine host than in culture. The other possibility is that during *in vitro* culture one type grows more successfully than the second which is lost.

From calf 170, infected with the *TaTu* 9A cell line, only two out of four variants

originally present in the parental stock (as assessed by probe *TaT* 17) were detected in both the piroplasms and the schizont-infected cell line (Table 6.9). This shows that particular variants might lose their infectivity after being cultured *in vitro*. Therefore the weakness of this study is the difficulty in obtaining, *in vitro*, cultures representing all the populations originally present in one isolate and to the limited amount of homologous piroplasms which can be obtained from field isolates.

The results indicated that more than one marker is required to detect the full level of diversity within a stock. For example stock *TaTu* 49 with a single homogeneous GPI profile, clearly consisted of several genotypes when DNA probes were used (Table 6.1). Likewise when DNA from stock *TaTu* 13, was probed with either *TaT* 17 or *TaT* 21, the piroplasms and schizont-infected cell line showed identical profiles, but GPI analysis of this stock demonstrated differences in that the piroplasm population was of a single profile, type 4, while the schizont-infected cell line was of single profile, type 6 (Table 6.8). On this basis the number of genotypes present has almost certainly been underestimated.

Bands detected by GPI electrophoresis or Southern blots (Fig. 6.3) were not always of equal intensity; such variation was seen between and within the isolates. Two explanations could be put forward: either the proportions of distinct parasite genotypes within the total population were different at the time of isolation or, in the case of schizont-infected cell lines, some of the genotypes overgrow others in culture and hence are represented by a larger proportion in the cell line. Indeed the study of Wilkie *et al* (1986) has shown that an equal parasite mixture of three different GPI type was found to be composed by only one subpopulation after extended passage *in vitro*.

The distribution of the different variants in the stocks could have been affected by selection in parasite populations during the establishment of the cell line *in vitro*. Ideally the study would have included an examination of the tick populations on the farms as performed by GPI analysis for the Chergui farm. But the selection *in vitro* appeared to only affect a minority of the stocks and this problem could not be foreseen before the study was planned. Moreover the use of tick material was difficult as ticks could often not be found in the farms where the disease occurred. If ticks were the only source of parasite the number

of samples would have been smaller than in this study and this could have reduced the number of different variants detected, especially those found less frequently.

Each method of sampling has its advantages and disadvantages and, depending on the situation and the means available, one or the other would be more appropriate. In this study, the polymorphism found and the number of variants detected is probably an underestimation of the real situation in the total cattle population in Tunisia.

The polymorphism between and within *T.annulata* stocks showed no correlation with the geographic location of the isolates, with differences observed even amongst those made from animals on the same farm. Similar results have been reported for *P.falciparum* isolates from patients in one village where even isolates from the same family were of different genotypes (Babiker *et al*, 1992b). The results also raise a concern for future studies in that the possibility of selection occurring through manipulation *in vitro* or *in vivo* should always be recognised when interpreting data to characterise *T.annulata* parasites. As large numbers of clones have not been established from each isolate it was not possible to determine the numbers of distinct genotypes within the population but, if the four markers used are unlinked, there may be a very large number within an individual animal. These results are in contrast to the findings of Matsuba *et al* (1992), on *T.sergenti*, who reported identical genomic patterns using two DNA probes for piroplasms DNA preparations from animals grazing in the same area, although polymorphism was observed between stocks isolated from different areas. The selection described here could also account for the disappearance of a genomic fragment in Southern blots, when stocks are passaged *in vivo* in the study of Matsuba *et al* (1992).

Diversity within individual parasite populations would seem to be an inherent facet of infection by *T.annulata* and has important implications for a successful vaccination programme using either a live attenuated or recombinant subunit vaccine, if the antigen generating immunity showed a similar level of diversity.

The next step in our investigation will be to provide an explanation for such diversity. Since genetic recombination occurs in *P.falciparum* (Ranford-Cartwright *et al*, 1991) and has now been reported for *T.parva* (Morzaria *et al*, 1992b) it is likely that it

might generate part of the considerable polymorphism seen in *T.annulata*. To examine this question further, individual clones and mixtures of clones were used to infect calves, ticks fed on them and the parasite populations developing in these ticks analysed and compared with those present in the calves. The results obtained and possible explanations for these are presented in the next chapter.

## ***CHAPTER SEVEN***

***INFECTIVITY, DIVERSITY AND GENETICS OF THEILERIA ANNULATA***



## 7.1 INTRODUCTION

The previous chapter described the extensive polymorphism detected in *T.annulata* stocks isolated within Tunisia. The study examined over 50 isolates and used four markers to analyse both genotypic and phenotypic variability. Considering the four markers together, no two isolates were identical. Even those isolated from animals on the same farm were different. Furthermore, many different combinations of the alleles at these loci were identified in clones established from cell lines infected with these stocks.

These results raise a number of questions of both fundamental and practical significance. These are as follows:

1. Is the ability of individual cloned subpopulations to infect both vertebrate and invertebrate hosts different from that of a pool of the same clones?
2. If a mixture of clones proves to be infective, yet individual clones are not, is this due to synergy, potentiation or simply the inclusion of a combination of strains?
3. Can protection against a heterologous lethal sporozoite challenge be provided by individual clones, or is a mixture of cloned lines necessary for protection?
4. If cloned cell lines are protective, do different clones have the same ability to protect?
5. Is continuous prolonged passage a necessary step towards the development of an attenuated vaccine or will clonal selection suffice? If clones can be selected that are avirulent, or "attenuated", this would avoid the long period of attenuation. At present, 2-3 years are necessary for the development of cell culture vaccines against tropical theileriosis.
6. Finally, does the parasite undergo a sexual phase leading to genetic exchange and recombination which would explain the diversity detected within the theilerial

population, or does asexual reproduction based on clonality account for the phenomenon observed?

An attempt to answer these questions was made by designing an experiment (Fig. 7.1) to study the infectivity of different clones and their mixtures for the vertebrate and invertebrate hosts, to analyse the variation observed between the parasites obtained from different life-cycle stages and to demonstrate recombination in *T.annulata*. The experiment conducted involved three main steps:

- (i) The infection of a series calves *in vivo* with cloned infected cell lines and with a pool of the same cloned lines;
- (ii) The feeding of ticks on the infected calves to pick up circulating parasites;
- (iii) The challenge of these calves with a lethal tick stabilate.

The cloned cell lines in this study were characterised using four markers of three types: a MAb, the isoenzyme GPI and two genomic probes (as described in Chapter 6). When determining which cloned cell lines to use, the clones which could be simply distinguished by Southern hybridisation using the probe *TaT 17* were chosen. Probe *TaT 17* identified five different genotypes of *T.annulata* which were designated 1 to 5 according to the size of the fragments recognised in Southern blotting (see Chapter five, Section 5.3.2). The five clones were isolated by limiting dilution from *T.annulata* infected cell lines (see Chapter 5, Section 5.3.2).

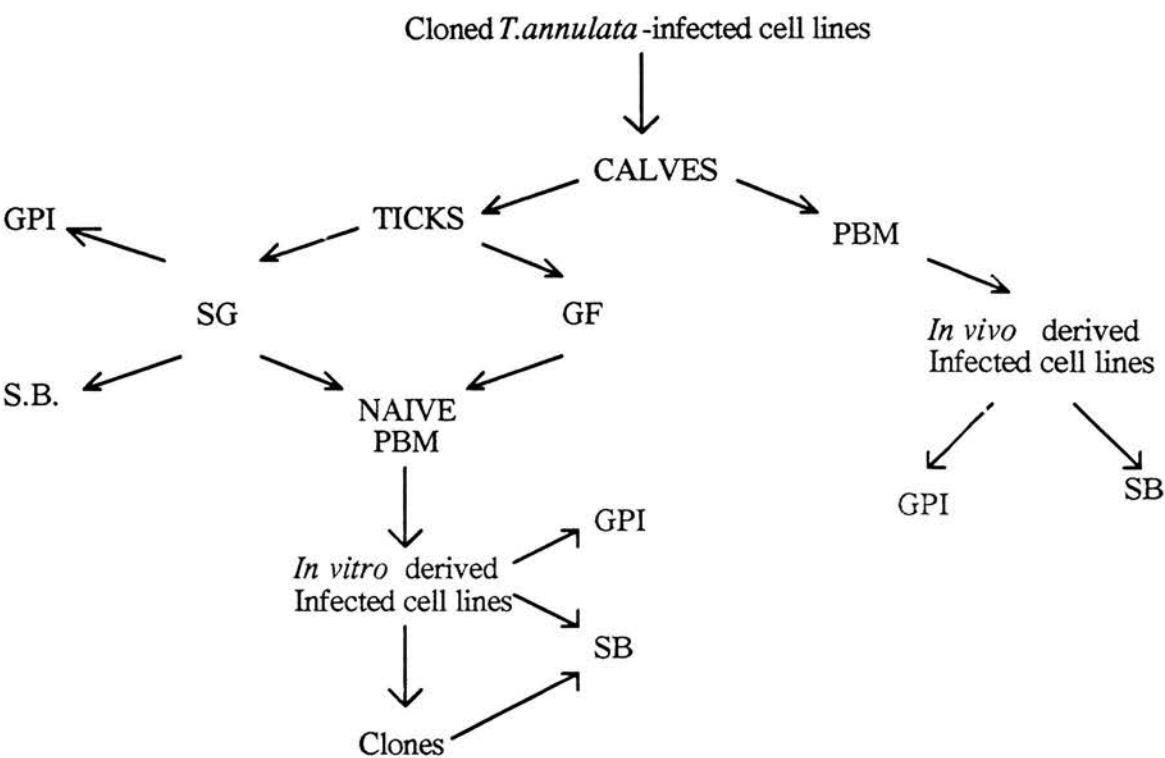
## **7.2. INFECTIVITY AND VIRULENCE**

### **7.2.1 *Infection of calves with cloned T.annulata infected cell lines***

#### **7.2.1.1 Experimental protocol**

The materials and methods used to infect calves were described in Chapter 2, and are briefly summarised here.

**Figure 7.1** Protocol of the experiment conducted in Chapter 7



- SB: Southern blotting
- SG: Salivary glands
- GF: Guts filtrate
- PBM: Peripheral blood mononuclear cells
- GPI: Glucose phosphate isomerase isoenzyme electrophoresis

a) Animals

Nine Holstein-Friesian male calves, aged 3-4 months, were assigned to two groups of five and four animals respectively. Calves in the first group were each inoculated individually with one of the five cloned cell lines while animals in the second group each received a mixture of the five clones in equal proportions.

b) Cell lines used and derived clones

The characteristics of the five cloned cell lines used to infect the calves are presented in Table 7.1.

c) Dose and route

The nine animals were inoculated with  $2 \times 10^6$  cells, from a 24 hr-old growing culture, subcutaneously over the left prescapular lymph node. The first group was inoculated with individual clones; 1, 2, 3, 4 and 5 respectively into animals 330, 600, 660, 290 and 370. Animals in the second group, calves 320, 360, 380 and 430, were each inoculated with an equal mixture comprising  $0.4 \times 10^6$  cells from each of the five clones.

d) Monitoring of the animals

The animals were examined clinically and parasitologically over a period of two months as described in Chapter 2. This included rectal temperature, haematology (PCV and WBC) and parasitology (schizonts in LN biopsies and piroplasm percentages in blood smears). The biological indicators measured for each calf are presented in the annexed charts. As markers of virulence and infection the following parameters were measured: maximum parasitaemia, minimum PCV, minimum total WBC count, number of days with a rectal temperature above  $39.5^{\circ}\text{C}$ , the presence of schizonts in LN biopsies and the presence of anti-schizont antibodies at day 30 of infection. In addition the presence of sporozoite-infected acini in the salivary glands of adult ticks derived from the nymphs fed on individual calves was determined.

The infection records from calf 400, infected with a cell line derived from a field isolate (see Chapter 6, Section 6.3), are given for comparative purposes. This calf was not challenged and the minimum PCV was observed on day 32 of infection. Therefore the minimum PCV for all calves was determined within the period of 35 days after infection with

**Table 7.1**      Characteristics of the five cloned cell lines used to infect the calves.

<b>Clone Type</b>	<b>Parental Cell Line</b>	<b>MAB 7E7 Reactivity</b>	<b>GPI Type</b>	<b>TaT 17 Profile</b>
Clone 1	<i>TaTu 13</i>	100%	6	1
Clone 2	<i>TaTu 9B</i>	100%	4	2
Clone 3	<i>TaTu 9B</i>	100%	2	3
Clone 4	<i>TaTu 9A</i>	100%	2	4
Clone 5	<i>TaTu 2</i>	0%	4	5

the cell lines. Challenge of the nine calves was undertaken on day 30 and it was thought that a fall of PCV five days after the challenge was not likely to be due to the latter but rather due to the initial infection. The details of all PCV's registered are presented in the annexed charts.

e) Ticks

One thousand nymphs of *H.a.excavatum*, were fed on each calf on day 19 of infection.

#### 7.2.1.2 Results

The individual results registered from the nine calves, together with comparative records from calf 400 are presented in the annexed charts. A summary of these results is given in Table 7.2. Calf 600 did not show any pyrexia and a minimum of three days of raised temperature was registered in the other calves. Two of the calves, 360 and 430, infected with the pool of clones exhibited temperatures above 39.5°C for 9 and 15 days respectively. It can be seen that overall the parasitemia in the nine calves inoculated with cloned cell lines varied between 0-1.5%. Calf 400 showed a maximum of 15% parasitemia.

The minimum PCV recorded for the nine calves varied between 27-17. The PCV of calf 400 infected with the field isolate fell to 15.

The minimum WBC count registered was for calves 430 and 660 and was  $4.6 \times 10^6$  cells/mm<sup>3</sup>.

Although schizonts were not detected in LN biopsies from calves 430, 290 and 400, infected cell lines were established from their PBM, as they were from calves in which schizonts were seen in LN biopsies.

Twenty ticks (10 male and 10 female) were dissected from batches of ticks collected from each animal after moulting, to assess the infection rate (proportion of ticks infected) and mean number of infected acini (see below in Table 7.4). The results showed that all calves, except calf 600, inoculated with clone 2, were infective for the invertebrate host. This correlates with the fact that no parasites, either schizonts or piroplasms, were found and no cell line could be established from the PBM of this calf. Additionally this animal did not show a single day of temperature above 39.5°C. Results of the IFAT, using schizont antigens, and sera from the calves at day 30 of infection with cell lines, also showed that calf 600 did not

**Table 7.2** Parameters of virulence considered during initial infections of calves with cloned infected cell lines: Immunisation

Calf's number	330	600	660	290	370	320	360	380	430	400
Type of clone(s) inoculated	1	2	3	4	5	Pool of the five clones				Field Isolate
Maximum Parasitemia (%)	0.1	0	0.1	<0.1	0.1	0.2	1	0.2	1.5	15
Minimum PCV	22	24	24	20	19	21	27	21	17	15
Minimum WBC count (x 10 <sup>6</sup> /mm <sup>3</sup> )	8	7.4	4.6	6.1	6.7	6.6	7.8	4.8	4.6	6.2
Number of days with temperature above 39.5°C	3	0	4	7	6	5	9	4	15	7
Presence of schizonts in LN biopsies	+	-	+	-	+	+	+	+	-	-
Establishment of cell lines from PBM <i>in vitro</i>	+	-	+	+	+	+	+	+	+	+
Tick infectivity	+	-	+	+	+	+	+	+	+	+
IFAT (1/160)	+	-	-	+	+	+	+	+	+	+

have detectable levels of anti-schizont antibodies. No antibodies to schizonts were found for calf 660, although an infected cell line was established from the PBM of this calf (Table 7.2).

The results indicated that, with the exception of clone 2, inoculated into calf 600, the cloned infected cell lines were able to infect calves.

The only clear difference between the two groups of animals, infected either with individual clones or with a pool of these, was the level of parasitemia. The maximum parasitemia recorded for an animal within the first group was 0.1% (Average less than 0.08%) while it reached 1.5% (Average = 0.725%) in the second group. The minimum parasitemia registered in animals of the second group was 0.2% which represented twice the maximum parasitemia of the first group. This would suggest that the mixture of clones were more effective than the individual clones comprising them. This was later supported from the results obtained from the analysis of the infection in the ticks (see below).

#### 7.2.1.3 Discussion

The severity of reaction can be determined by a number of factors such as the summation of infectivity and virulence of the parasite and the individual animal response to that inoculation which often might indicate that one animal is more affected by the parasite than another.

There was no consideration of an MHC barrier when infecting the calves with cell lines on the basis of previous studies which have shown that MHC does not interfere with the infectivity of *T.annulata*-infected cells. (Innes, 1988; Innes *et al*, 1992).

The first aspect considered during this trial was if infection was established or not in both the calves infected with individual clones and those infected with the pool of these clones. The results showed that all clones, except clone 2, were able to infect the calves. It was confirmed as discussed later using Southern blot analysis that this clone also failed to establish in the four calves infected with the pool of clones. This result might only be specific to clone 2, which could have totally lost its ability to infect calves, but the correlation between the specific genotype of this clone (and of any others), as defined by probe *TaT* 17, and the



apparent infectivity of the clone, can only be assessed when similar genotypes cloned from other parental cell lines are tested.

The second aspect considered was the virulence of the material used to infect the calves. This was analysed using the severity and duration of the fever period, the maximum parasitemia, the minimum WBC count and the fall in PCV recorded for each of the calves. Statistical analysis could not be conducted because of the nature of the data (non-parametric for some of them) and the number of animals included in each group (5 and 4 respectively). However, the general trends were that animals infected with the mixture of clones developed more severe reactions than those infected with individual clones. Indeed the analysis of the results showed that the fever period was more severe, the parasitemia higher and the fall in PCV greater in the animals which received the pool of clones.

The results of this trial also showed that the infection with cloned *T.annulata* infected cell lines, whether individually or as a mixture, at this dose level, is well tolerated by the calves. There was no major sign of serious disease in any calf, although in some animals a temperature above 39.5°C was recorded for more than three days. Calves 360 and 430 were both febrile when the experiment started (see annexed charts) and this probably accounts, to some extent, for the duration of high temperatures registered for these calves (9 and 15 days respectively) during the first week of infection.

The fever period, however, should be seriously considered, since this might affect animal productivity if such cell lines are to be considered as potential vaccines.

### **7.2.2 *Sporozoite challenge of the calves***

This part of the trial was conducted for the following reasons:

- (i) To confirm the infectivity of the parasite material used.
- (ii) To select appropriate clones as an approach to making a vaccine.
- (iii) To study the protection conferred either by the individual clones or the mixture of them.

#### **7.2.2.1 Experimental protocol and results**

The nine calves were challenged on day 30 after infection with the cloned cell lines, with 1.6 te of *TaTu* 9A sporozoite stabilate derived from *H.a.excavatum* nymphs, subcutaneously over the right prescapular lymph node (Chapter 2, Section 2.2.3.6). The vials were taken from liquid nitrogen, thawed, then left to equilibrate at ambient temperature (30-35°C) for 15 min. They were pooled then transported from the ENMV to the IPT farm over the next 30 min at ambient temperature (which might have reached 50°C inside the car used for transport). The sporozoites were inoculated with a 2ml syringe and 21 gauge needle. Each animal was infected over the following 30 min, a total time of 90 min elapsing between thawing the stabilate and inoculating the last calf.

The results of the reaction to challenge are given in detail in the annexed charts for each calf, and are summarised in Table 7.3. All calves were infected by this stabilate challenge, showed piroplasms in blood smears and *T.annulata*-infected cell lines were established from each of the nine calves. All calves also had a positive titre (1/160) to schizonts in IFAT at day 30 after challenge.

The first piroplasms do not appear in blood smears before day 9 following infection with a sporozoite tick stabilate (C.G.D.Brown, unpublished data). Therefore only parasitemias recorded after day 7 of challenge were considered to be related to the challenge infection.

As shown in Table 7.3, the maximum parasitemia recorded in the group of calves immunised with the pool of clones was 0.2% (calf 320). The number of days where fever was recorded varied between 0-10 days in this group while the PCV and WBC count did not fall markedly. The animals underwent mild reactions which were only clinically obvious in calves 320 and 430 which had fever for 5 and 10 days respectively. From the parameters studied, by comparison with the other three calves in the group, calf 430 reacted the most severely to both immunisation and challenge inoculations (Tables 7.2 and 7.3).

The maximum piroplasm parasitemia recorded in the group of calves immunised with individual clones was 5%, in calf 290, while the minimum PCV and WBC count in animals of this group was respectively 19 and 4.6, registered in calf 600. It is from these calves that the maximum numbers of days with fever were recorded. They were of 5 and 6 days respectively.



#### 7.2.2.2 Discussion

No control animals were included in this trial since the stabilate had already proved to be fully virulent, though under different conditions. However, calf 600 which did not show any apparent reactions to immunisation and was assumed not to have been infected by the parasite, i.e. the parasite did not develop *in vivo*, was considered as internal control to the trial. It was expected that calf 600, would react severely to the challenge if not die. This calf showed fever, parasitemia and the lowest PCV and WBC count of the group, but no serious disease. This showed that it was infected with the challenge parasite material but also indicated that the challenge was relatively mild. Overall the reactions in the challenged calves, as described, were not significant. This is in marked contrast to reactions seen in experiments conducted at the ENMV on calves inoculated with cell lines derived from field isolates and challenged with the same stabilate on other occasions (Dr M.Darghouth, unpublished data). It could be interpreted that the sporozoites, or a porportion of them, were viable but might have suffered during transport from excessive heat, resulting in relatively mild challenge rather than a lethal one due to a serious reduction in the sporozoite infectivity. Therefore it is difficult to draw final conclusions in relation to the results of challenge. However a few useful observations could be made from this trial.

The maximum parasitemias and minimum PCV, showed that the reactions to the challenge were slightly more marked in the first group of calves, immunised with individual clones, than in the second group, where animals were inoculated with the pool of five clones. This might have resulted from either a greater level of immune response in the second group since it showed a more marked reaction to the initial infection or from the portfolio of antigens to which the second group had been exposed when inoculated with the pool of cell line clones. However, the severity of the challenge in no way was affected by the severity of the primary response.

The duration of the fever in animal 430 was 10 days, even though two were recorded before day 5 of challenge and therefore might be due to the initial infection, indicating again that this animal was more susceptible, to theilerial infections. This was observed whether it was immunised or not, since a high parasitemia (1.5%) and a marked fall in PCV (17) were

observed during the initial infection but not during challenge. The reactions observed for this animal were unlikely related to the parasite itself since each calf from the group received the same parasite material during immunisation and challenge. This individual susceptibility to *Theileria* infection is an important factor to consider during vaccination programmes.

### **7.2.3 Infectivity for the invertebrate host**

#### **7.2.3.1 Experimental protocol**

One thousand nymphs of *H.a.excavatum* were fed on each calf reacting to the immunisation with the cloned cell lines (not to the challenge). After moulting, they were incubated at 37°C for four days to induce the maturation of sporozoites.

Twenty ticks (10 male and 10 female) from each batch were dissected and the salivary glands stained with MGP to assess the infection rates and the mean number of infected acini.

#### **7.2.3.2 Results**

The results for this section are presented in Table 7.4. Final yields of adults ticks of each batch are shown. It could be seen that variable number of ticks dropped (90-540) and survived to moult (67-450) from the calves.

Infection rates in the ticks ranged between 0-100% while the mean numbers of infected acini per tick varied between 0-403.9. Differences were noticed between the two groups of calves. Ticks fed on calves infected with individual clones showed a mean infection rate of 16%. Those fed on calves infected with the pool of clones showed a mean infection rate of 73.5%. The average of means number of infected acini per tick was also markedly different in the two groups. It was of 0.91 for ticks fed on calves infected with individual clones and of 196.35 for ticks fed on calves infected with the pool of clones.

#### **7.2.3.3 Discussion**

The analysis of the infection rates and mean numbers of infected acini confirmed the observations *in vivo*, in that the calves infected with the pool of clones were more affected by the parasites than the ones infected with individual clones. Specifically, they exhibited a

**Table 7.4** Feeding performances and infection rates of *Hyalomma excavatum* ticks fed on calves infected with pooled and individual clones of *T. annulata*-infected cell lines (1000 nymphs were applied to each calf).

Calf's number	330	600	660	290	370	380	320	430	360
Type of clone used to infect calves	1	2	3	4	5	Pool of the five clones			
Nymphs collected after feeding	200	90	540	460	220	330	400	178	320
Adults collected after moulting	67	75	450	400	150	280	340	170	235
Infection rate (%)	15	0	10	5	50	5	95	100	95
Mean number of infected acini per tick	0.55	0	0.15	0.05	3.8	0.1	25.9	353.5	403.9

markedly higher parasitemia whose infectivity for ticks was exemplified by these results. Tick infection rates were much higher for the former group (Table 7.4). It could be argued that the parasitic populations of *T.annulata* have different abilities to infect ticks and that, when they are mixed, the clones which have better potential to infect the vector facilitate the infection by the other clones that might be defective on their own. However, other unknown factors might also intervene since not all ticks fed on calves inoculated with the pool of clones did show high infection rates. Indeed, ticks fed on calf 380, had only an infection rate of 5% as compared to 95-100% in the three batches of ticks fed on the remaining calves infected with the pool of clones.

The relatively low number of ticks collected after feeding and moulting could be explained by different susceptibility of the calves to tick infections in the first case and probably differences between the batches of ticks in the second case since they were not obtained from the same female (M.Darghouth, personal communication).

### **7.3 DIVERSITY OF THE PARASITE MATERIAL OBTAINED**

#### **7.3.1 *Experimental protocol***

The protocol is described in Fig. 7.1. The *H.a.excavatum* tick were used to prepare sporozoite suspensions for GF (see Chapter 2, Section 2.2.4.3). GF from each of the nine batches of ticks were used to infect PBM from a naive single donor, calf 260. Infections *in vitro* were also performed from three individual ticks fed on calf 320, using dissected salivary glands (Chapter 2, Section 2.2.4.1).

All cell lines established were studied using GPI electrophoresis and Southern blotting with probe *TaT* 17. They were not examined by MAbs and IFAT.

Sporozoite lysates for DNA extraction and GPI electrophoresis were also prepared from salivary gland extracts.

#### **7.3.2 *Results***

##### **7.3.2.1 Tick salivary gland lysates**



Analysis of lysates was only performed using preparations from salivary glands of ticks fed on calves 430 and 360 both of which had been inoculated with the pool of five clones. They showed both high infection rates and exhibited large number of acini infected. The GPI analysis showed that both lysates comprised subpopulations of type 2 and 4 profiles (data not shown) (Table 7.5). Probe *TaT* 17 showed the presence of subpopulations of clone 1, clone 3 and clone 5 types for calf 430 and of clone 1, clone 4 and clone 5 types for calf 360 (Fig. 7.2, tracks 1 and 2; Table 7.5).

#### **7.3.2.2 Schizont-infected cell lines derived from *in vitro* infections.**

Infections *in vitro* from the four batches of ticks fed on calves infected with the pool of clones were all successful, with infected cell lines becoming established. Only two cell lines were established *in vitro* from the five batches of ticks fed on calves infected with individual clones. These were obtained from batches of ticks fed on calves 290 and 370. This was despite repeated attempts to establish cell lines from batches of ticks fed on calves 600, 330 and 660.

The six derived infected cell lines, designated by the number of the calf from which the parasites were derived followed by the one of the PBM donor (calf 260), were analysed by GPI electrophoresis and Southern blotting using probe *TaT* 17. The two cell lines deriving from infections with individual clones were identical to the original clones as assessed by both GPI (data not shown) and Southern blotting (Fig. 7.2, tracks 11 and 12) (Table 7.5). The results of the analysis of the remaining cell lines established from ticks fed on calves infected with the pool of five clones are presented in Table 7.5. All cell lines of this group showed the presence of subpopulations of type 2 and type 4 in GPI electrophoresis analysis (Fig. 7.3, tracks 1, 3, 5, 7). Cell line *TaTu* 380/260 presented an additional population of type 6 (Fig. 7.3, track 5). The results from Southern blotting, using probe *TaT*17, showed the presence of subpopulation of clone 1 type in cell line *TaTu* 380/260 in addition to subpopulations of clone 4 and clone 5 types. These last two types were the only subpopulations detected in cell lines *TaTu* 320/260 and *TaTu* 360/260 whereas cell lines *TaTu* 430/260 showed subpopulations of clone 3 and clone 5 types (Fig. 7.2; Table 7.5).



**Table 7.5**      Characteristics of the cell lines obtained from the infection of the calves with cloned cell lines

Types of clone(s) innoculated	Calf's Number						
		290	370	320	360	380	430
	GPI	2	4	2 + 4 + 6			
	TaT 17	4	5	1 + 2 + 3 + 4 + 5			
Type of profiles obtained from the cell lines established from the ticks	GPI	2	4	2 + 4	2 + 4	2 + 4 + 6	2 + 4
	TaT 17	4	5	4 + 5	4 + 5	1 + 4 + 5	3 + 5
Types of profiles obtained from salivary gland extracts	GPI	-	-	-	2 + 4	-	2 + 4
	TaT 17	-	-	-	1 + 4 + 5	-	1 + 3 + 5
Types of profiles obtained from the cell lines established from the calf's PBM	GPI	2	4	2	4	2	2
	TaT 17	4	5	3 + 4	5	3 + 4	3
Types of profiles obtained from cell lines established from individual salivary glands	GPI	-	-	C1 C2 C3			-
				2 + 4			
				4	3	4	
				+	+	+	
	TaT 17	-	-	5	5	5	-

**Figure 7.2** Southern blot of *T.annulata* digested with *Eco*RI and hybridised with probe *Ta*T 17. Tracks correspond to stocks.

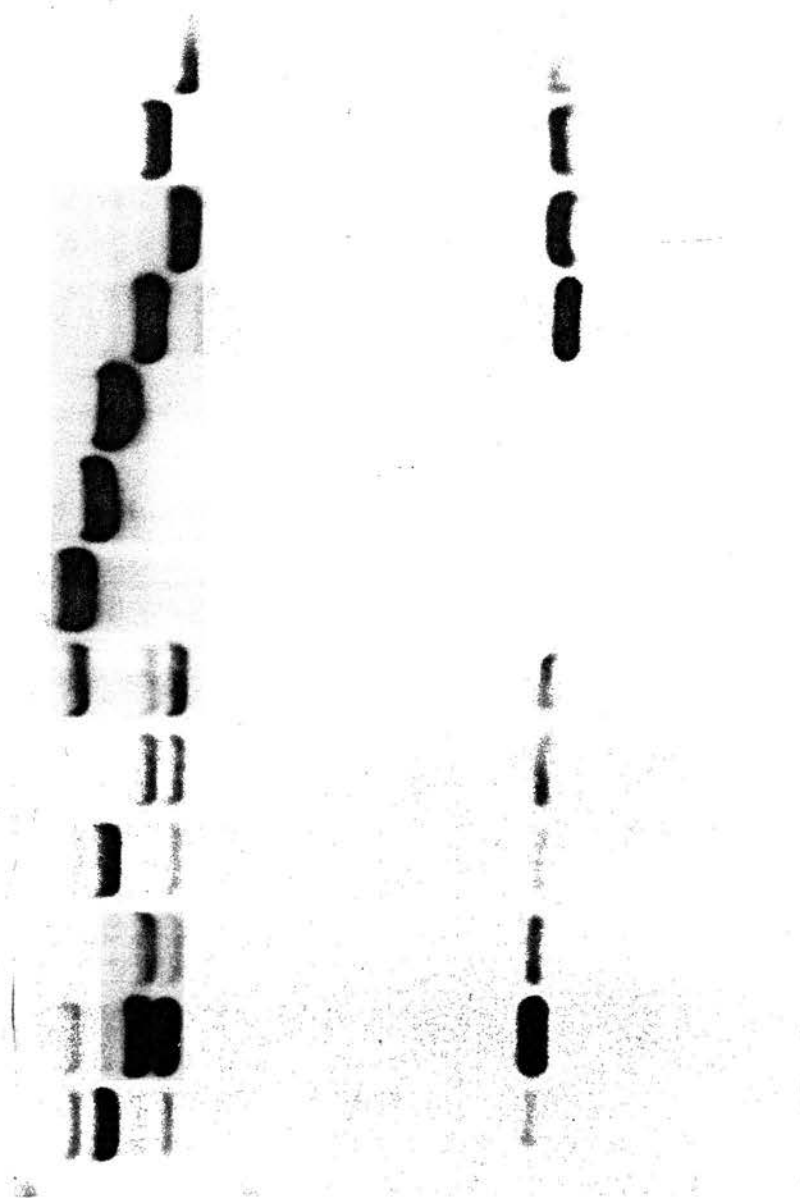
Tracks	Stocks
1	<i>Ta</i> Tu 430 sporozoites
2	<i>Ta</i> Tu 360 sporozoites
3	<i>Ta</i> Tu 360/260 cell line
4	<i>Ta</i> Tu 430/260 cell line
5	<i>Ta</i> Tu 320/260 cell line
6	<i>Ta</i> Tu 380/260 cell line
7	clone 1 cell line
8	clone 2 cell line
9	clone 3 cell line
10	clone 4 cell line
11	clone 5 cell line
12	<i>Ta</i> Tu 290/260 cell line
13	<i>Ta</i> Tu 370/260 cell line

Kb

8.6  
“ 7.6  
“ 6.8  
“ 5.6  
“ 4.9

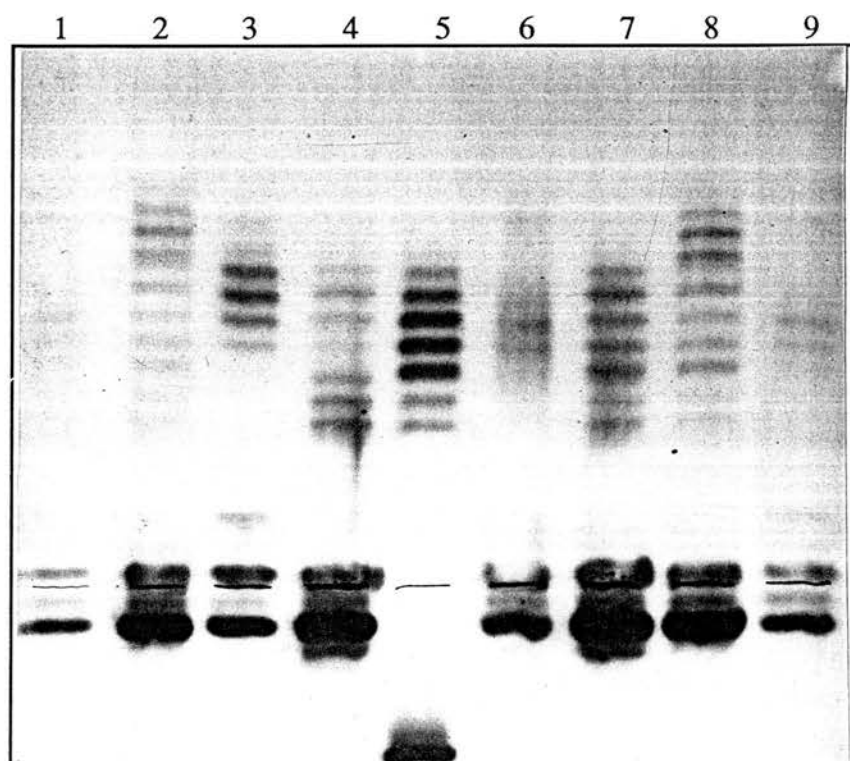
“ 1.8

1 2 3 4 5 6 7 8 9 10 11 12 13



**Figure 7.3** Comparison between GPI profiles of *T.annulata*-infected cell lines established from the calves infected with the pool of cloned cell lines and those obtained from cell lines established from the ticks fed on them. Tracks correspond to stocks.

Tracks	<i>T.annulata</i> -infected cell lines
1	<i>TaTu</i> 320/260
2	<i>TaTu</i> C320
3	<i>TaTu</i> 360/260
4	<i>TaTu</i> C360
5	<i>TaTu</i> 380/260
6	<i>TaTu</i> C380
7	<i>TaTu</i> 430/260
8	<i>TaTu</i> 430



In order to investigate if individual ticks carry the same set of parasite genotypes as a pool of infected ticks, three individual ticks from those fed on calf 320 were dissected and the individual salivary glands used for *in vitro* infection. Three cell lines were established, named C1, C2 and C3. Using GPI electrophoresis analysis, the three cell lines showed an identical pattern to that of *TaTu* 320/260 (data not shown). Analysis by Southern blotting, using probe *TaT* 17, showed that C1 and C3 were composed of subpopulations of clone 4 and clone 5 types (as found for *TaTu* 320/260) (data not shown) while C2 comprised subpopulations of clone 3 and clone 5 types (Fig. 7.4, track 1).

#### **7.3.2.3 Schizont-infected cell lines obtained from infections *in vivo*.**

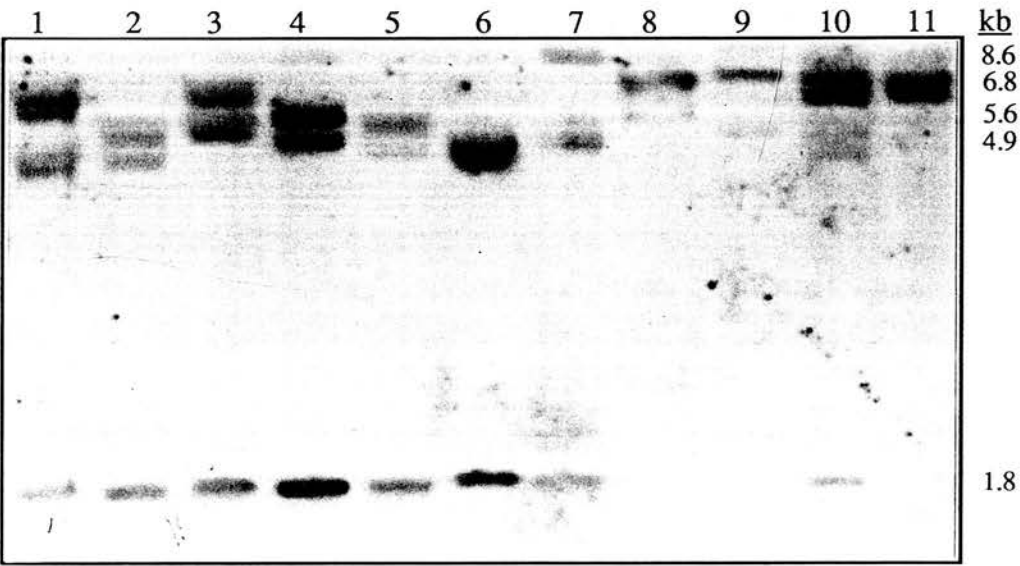
Cell lines established from PBM of the calves during immunisation and designated by the calf number, e.g. *TaTu* C330, were analysed using GPI electrophoresis and Southern blotting with probe *TaT* 17. The four cell lines obtained from each of the calves infected with individual clones (no cell line could be established from the fifth calf, calf 600) showed the same marker profile as the clone used to infect each calf (data not shown). Results obtained from the analysis of the cell lines established directly from the remaining four calves infected with the pool of clones are presented in Table 7.5. This illustrates that there were differences between the profiles detected in these *in vivo* derived cell lines and those established by *in vitro* infection with the sporozoites harvested from the ticks fed on these calves as individuals. The GPI electrophoresis and DNA analyses illustrating these differences are shown in Figs. 7.3 and 7.4 respectively.

#### **7.3.3 Discussion**

This section is primarily intended to assist in the analysis of the diversity of cell lines derived from *in vitro* and *in vivo* infections. Differences in the populations defined by GPI analysis and Southern blotting between the cell lines established from infection *in vitro* using sporozoites and those established from PBM of the calves during the initial infection with cell lines, were noticed. The latter showing more restricted profiles than the former. This could be due to parasite selection *in vitro* during the establishment of the cell lines from the PBM as

**Figure 7.4** Southern blot of *T.annulata*-infected cell lines digested with *Eco*RI and hybridised with probe *Ta*T 17. Tracks correspond to stocks.

Tracks	<i>T.annulata</i> -infected cell lines
1	C2
2	<i>Ta</i> Tu 320/260
3	<i>Ta</i> Tu C320
4 and 5	<i>Ta</i> Tu 360/260
6	<i>Ta</i> Tu C360
7	<i>Ta</i> Tu 380/260
8	<i>Ta</i> Tu C380
9 and 10	<i>Ta</i> Tu 430/260
11	<i>Ta</i> Tu C430





discussed in Chapter 6 (Section 6.4), as some of the genotypes were only observed in the cell lines established from the same calves using sporozoites e.g. clone type 5 in *TaTu* 430/260, *TaTu* 380/260 and *TaTu* 320/260 (Table 7.5). In contrast the population represented by clone 3 was detected in the cell lines established from PBM of calves 320 and 380 but not in their respective cell lines obtained from the infections *in vitro* with the pool of ticks (GF). This might be due to the fact that this clone is a poor producer of merozoites and therefore piroplasms *in vivo*, as shown for 'diminished clones' *in vitro* (Shiels *et al*, 1992). In this case the chance of the population representing this clone being picked by ticks would be disadvantaged compared to highly producing merozoite populations ('enhanced clones' *in vitro*, Shiels *et al*, 1992). It could, however, still be easily isolated from the PBM of the calf, as a cell line derived directly from the schizont stage of the parasite. Accordingly, when individual ticks, fed on calf 320, were used to establish cell lines *in vitro*, the population corresponding to clone 3 was detected in the cell line C2. This probably means that this population was represented at a very low level in the tick population fed on calf 320 and was either not able to be established *in vitro* from the pool of ticks or was established but not detected, or was established, but other populations overgrew it during culture.

Another possibility is that this population has a better ability to develop in the calf *in vivo* than to infect the vector tick, since it is found in three calves out of four and only in one cell line out of the four cell lines established from the pool of ticks (*TaTu* 260/430). This clone did infect calf 660 when used alone. But although the infection rate of the ticks fed on this calf (10%) was not lower than those of ticks fed on calves 290 (5%) or 380 (5%) (Table 7.4), no cell lines could be established from the ticks fed on it despite several attempts. In contrast, cell lines were readily obtained from ticks fed on calves 290 and 380 infected, respectively, with clone 4 and the pool of the five clones. These observations could mean that this clone has a poor ability to infect PBM *in vitro* or that the calf used (calf 260) as a donor of PBM showed particular resistance to this clone.

The analysis of the populations established from ticks fed on calves infected with the pool of clones showed that populations of clone 4 and clone 5 types were present in the highest frequency in all four animals. This, probably correlates with the fact that only these

populations were established from ticks fed on calves infected with individual clones (Table 7.4). Populations of clone 1 type or clone 3 type were established from one batch of ticks out of the four fed on calves infected with a mixture of clones, while such populations could not be established from ticks which were fed on calves infected with these type of clones individually. This might simply be due to the fact that, by infecting four calves, the chance of establishing a defined population is greater than when infecting only one.

The clones used to infect the calves, excluding clone 2, did not behave identically in the four calves, in the ticks fed on them, or in the cell lines obtained from the latter. Populations of clone 3 and clone 4 types were more easily obtained from the PBM of the infected calves, whereas populations of clone 5 and clone 4 types were more easily established in cell lines derived from infected ticks *in vitro*. Considering that each type of clone was put into five animals (in four calves together with the four other clones and in the fifth individually), and considering the three following phases of selection — the parasite populations established in the animal, the parasite populations picked up by the ticks and the parasite populations transmitted from the ticks to naive PBM *in vitro* — it is clear that clone 5 type was more successfully transmitted than the clone 4 type, while clone 3 type and clone 1 type were poorly transmitted. Clone 2 type was not transmitted at all under any circumstances.

## **7.4 RECOMBINATION AND SEXUAL REPRODUCTION**

### **7.4.1 *Experimental protocol***

One way to demonstrate sexual reproduction is to show genetic exchange and recombination (Walliker, 1989; Walliker, 1991). This is an alternative to a reproduction prevalingly clonal as inferred for some parasitic protozoa (Tibayrenc and Ayala, 1991). The occurrence of genetic exchange and recombination may also help to explain the great level of variation detected in parasite populations (Tait and Turner, 1990; Walliker 1991).

In order to demonstrate sexual reproduction parasite subpopulations of clearly defined profile using a number of markers, are mixed, used to infect the invertebrate vector, and the progeny cloned, and characterised with the same markers. Recombinant clones will appear as parasites exhibiting non-parental combinations of these markers.

In this study, two cell lines, *TaTu* 320/260 and *TaTu* 360/260, obtained from *in vitro* infection with sporozoites derived from ticks fed on calves 320 and 360, which had been injected with the pool of the five clones, were cloned by limiting dilution. Cloned cell lines, from each parental cell line, were expanded and DNA prepared from them and used in Southern blotting analysis.

Three DNA probes were used to characterise the progeny clones. Two already described in this study *TaT* 17 and *TaT* 21 and an additional probe kindly provided by Dr.B.R.Shiels (Veterinary Parasitology, Glasgow), *TaA* 30.

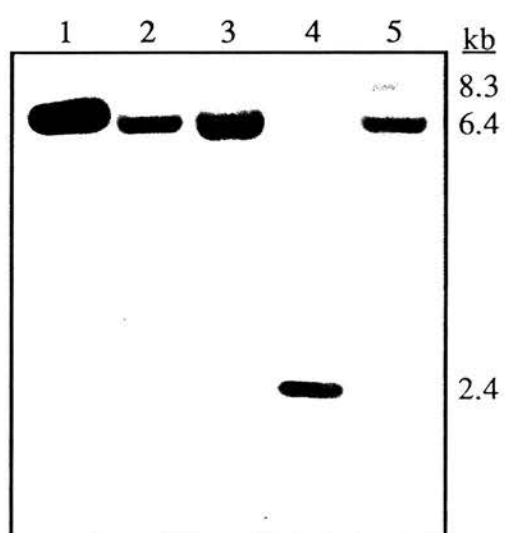
#### 7.4.2 Results

Results of the hybridisation of DNA from clones 1-5 (used to infect the calves) with probe *TaA* 30 are shown in Fig. 7.5. Clones 1, 2 and 3 presented a single fragment of 6.4Kb, clone 4 a fragment of 2.4Kb and clone 5 a combination of a 8.3Kb fragment and a 6.4Kb fragment.

Cell lines *TaTu* 320/260 and *TaTu* 360/260 and 10 clones derived from each were analysed by Southern blotting. The ten cloned cell lines derived from *TaTu* 320/260 when probed with *TaT* 17 and *TaA* 30 showed two clear profiles as illustrated in Fig. 7.6 (Tracks 2-11). The analysis of the clones using probe *TaT* 17 indicated that five of these were of clone 4 type (identified by the 5.6/1.8Kb fragments) (Fig. 7.6, A; tracks, 3, 4, 7, 9 and 10) whereas the remaining five clones were of clone 5 type (4.9/1.8Kb) (Fig. 7.6, A; tracks 2, 5, 6, 8 and 11). The same Southern blot was stripped then probed with *TaA* 30, showing the presence of the 6.4Kb fragment in seven clones (Fig. 7.6, B; tracks 2, 4, 5, 7, 8, 9 and 10) and the 2.4Kb fragment in three clones (Fig. 7.6, B; tracks 3, 6 and 11).

The subpopulation used to infect the calves originally, as defined by the two probes were of two types. The first was of clone 4 type which presented a 2.4Kb fragment detected by the *TaA* 30 probe and fragments of 5.6 and 1.8Kb detected by *TaT* 17. The second subpopulation was of clone 5 type which showed either the 8.3Kb fragment or the 6.4Kb fragment detected with probe *TaA* 30 (Fig. 7.5) and the 4.9 and 1.8Kb fragments detected by probe *TaT* 17.

**Figure 7.5** Southern blot of clones 1-5 digested with *Eco*RI and hybridised with prob  
*TaA* 30. Tracks correspond to clones.



**Figure 7.6** Southern blot of cell line *TaTu* 320/260 (Track 1) and ten derived clones (Tracks 2-11), digested with *Eco*RI and hybridised with probes *TaT* 17(A) and *TaA* 30(B).

Recombinant clones are seen in underlined tracks.

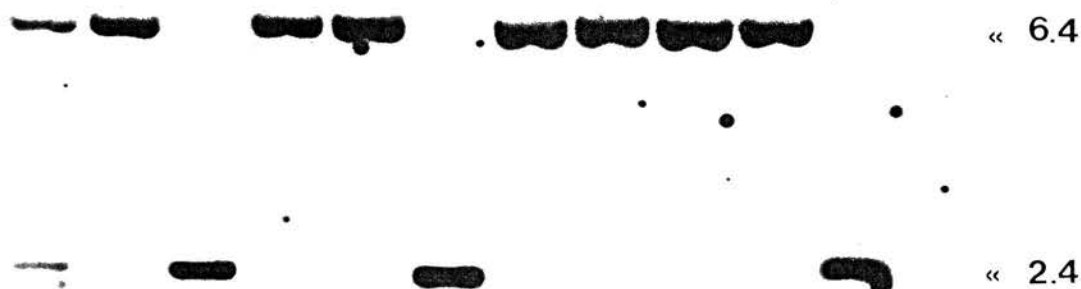
A

Kb



B

1 2 3 4 5 6 7 8 9 10 11



From the results, as summarised in Table 7.6, it could be seen that four clones showed parental combinations of characters (Fig. 7.6, tracks 2, 3, 5 and 8). One clone, clone II (Table 7.6) was of clone 4 type (5.6 and 1.8Kb, *TaT* 17) and showed the presence of the 2.4Kb fragment defined by probe *TaA* 30 (Fig. 7.6, track 3) and three others, clones I, IV and VII (Table 7.6) were of clone 5 type (4.9 and 1.8Kb, *TaT* 17) and showed the presence of the 6.3Kb fragment (*TaA* 30) (fig. 7.6; tracks 2, 5 and 8).

The remaining six clones, clones III, V, VI, VIII, IX and X (Table 7.6) (Fig. 7.6, underlined tracks) showed a recombinant genotype with one allele derived from one parental cell line and the other allele from the other parental cell line. Thus, they were either of clone 4 type (*TaT* 7) and showing the 6.3Kb fragment (*TaA* 30) (Fig. 7.6; tracks 4, 7, 9 and 10) or of clone 5 type and showing the 2.4Kb fragment (*TaA* 30) (Fig. 7.5, tracks 6 and 11).

Similar results were obtained from the analysis of the clones deriving from cell line *TaTu* 360/260. Of nine clones, four showed a clear recombinant genotype and five were of parental genotype, the 10th clone presented mixed genotypes (data not shown).

These clones were analysed with probe *TaT* 21 as well as with probe *TaT* 17 (Fig. 7.7, Table 7.7). It could be seen that clone II (Table 7.7), presented a combination of alleles that clearly demonstrates that it is a recombinant. It was also noticed that some of the clones presented mixed genotypes and therefore results from these could not be interpreted (clones IV, VI, Table 7.7). The remaining seven clones presented one or other of the parental profiles.

The analysis of the clones derived from *TaTu* 320/260 using the *TaT* 21 probe (in addition to *TaT* 17 probe) did not show any recombinant (data not shown).

Four chromosomes have been identified and separated by PFGE in *T.annulata* (Tait and Buchanan, unpublished data). Blots of such pulse field separations were probed with *TaT* 17, *TaT* 21 and *TaA* 30 to establish the chromosomal location of these probes. The results showed that *TaA* 30 is located on chromosome 1 (4.6Mb) while *TaT* 17 and *TaT* 21 are located on chromosome 3 (1.9Mb) (Tait and Buchanan, unpublished data).

### 7.4.3 Discussion

Based on morphological evidence, zygote formation and meiosis are presumed to



**Table 7.6**      Analysis of cell line *TaTu* 320/260 and derived clones using Southern blotting with probes *TaT* 17 and *TaA* 30

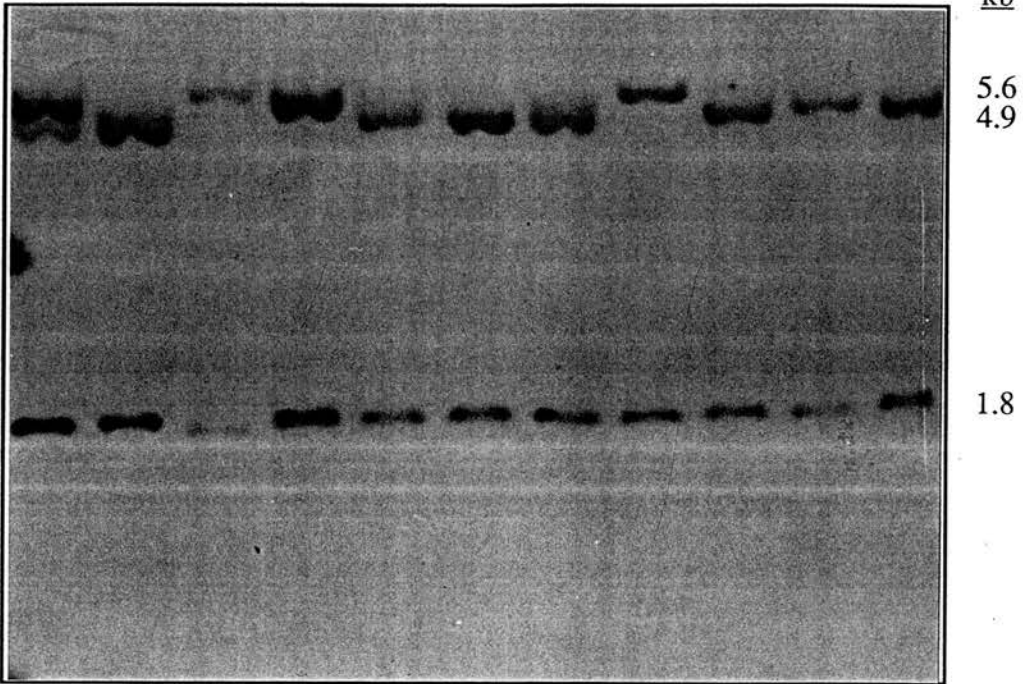
<i>TaT</i> 320/260	<i>TaT</i> 17	<i>TaA</i> 30	
Parental clone 4	5.6 + 1.8	2.4	
X			
Parental clone 5a	4.9 + 1.8	8.3	
b	4.9 + 1.8	6.4	
Clones			
I	4.9 + 1.8	6.4	Cl 5
II	5.6 + 1.8	2.4	Cl 4
III	5.6 + 1.8	6.4	R
IV	4.9 + 1.8	6.4	Cl 5
V	4.9 + 1.8	2.4	R
VI	5.6 + 1.8	6.4	R
VII	4.9 + 1.8	6.4	Cl 5
VIII	5.6 + 1.8	6.4	R
IX	5.6 + 1.8	6.4	R
X	4.9 + 1.8	2.4	R

R:      Recombinant

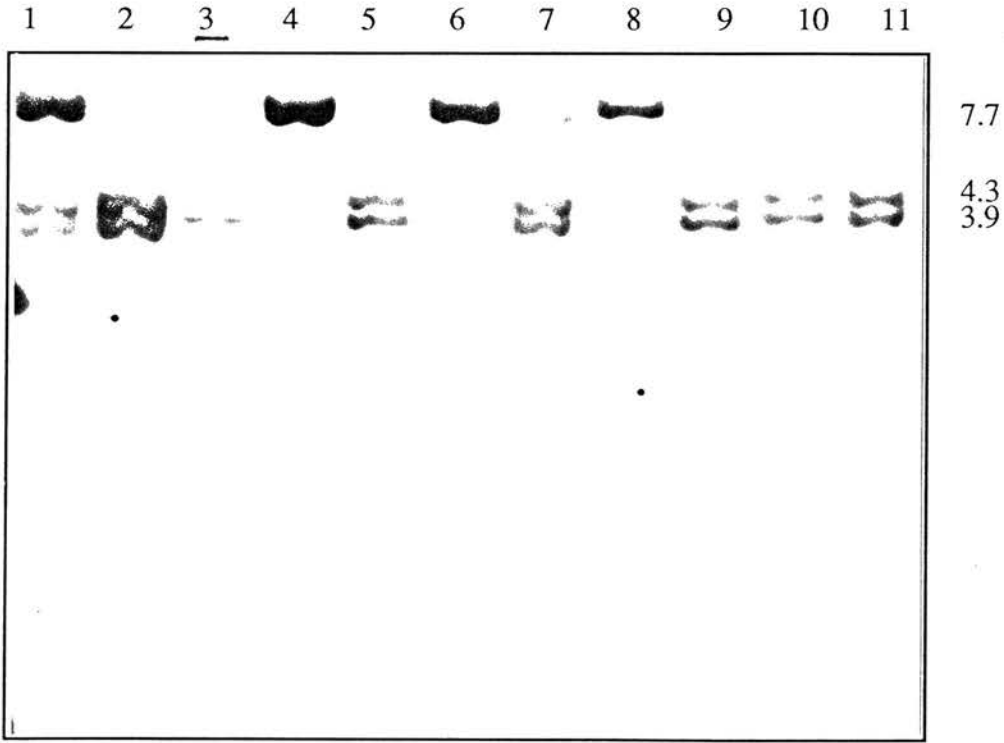
**Figure 7.7** Southern blot of cell lines *TaTu* 360/260 (Track 1) and ten derived clones (Tracks 2-11), digested with *Eco*RI and hybridised with probes *TaT* 17(A) and *TaT* 21(B).

Recombinant clone is seen in underlined track.

(A)



(B)



**Table 7.7**      Analysis of cell line *TaTu* 360/260 and derived clones using Southern blotting with probes *TaT* 17 and *TaT* 21

<i>TaT</i> 360/260	<i>TaT</i> 17	<i>TaA</i> 21	
Parental clone 4	5.6 + 1.8	7.7	
X			
Parental clone 5a	4.9 + 1.8	7.7	
b	4.9 + 1.8	4.3/3.9	
Clones			
I	4.9 + 1.8	4.3/3.9	Cl 5 b
II	5.6 + 1.8	4.3/3.9	R
III	5.6 + 1.8	7.7	Cl 4
IV	4.9 + 1.8	7.7 + 4.3/3.9	M
V	4.9 + 1.8	7.7	Cl 5 a
VI	4.9 + 1.8	7.7 + 4.3/3.9	M
VII	5.6 + 1.8	7.7	Cl 4
VIII	4.9 + 1.8	4.3/3.9	Cl 5 b
IX	4.9 + 1.8	4.3/3.9	Cl 5 b
X	4.9 + 1.8	4.3/3.9	Cl 5 b

R:      Recombinant  
M:      Mixed genotypes

occur in the tick gut (Schein and Friedhoff, 1978). While mitosis is a conservative process, maintaining the stability of genetic characters, meiosis is a process that generates enormous variation through independent assortment of chromosomes and crossing over (Suzuki *et al*, 1981). To investigate if such events do occur in *T.annulata* and therefore contribute to the variation seen in the population studied, analysis was conducted using polymorphic markers. In this study, five well defined cloned populations were used to infect calves, as a mixture, and circulating parasites were picked up by the vector tick *H.a.excavatum*. It was argued that, if genetic exchange occurred, it would result in the generation of recombinant parasites i.e. parasites with new combinations of alleles not present in the parental lines used to infect the calves.

To perform this analysis, the probe defining the five clones, *TaT* 17, and two additional probes were used. Additional markers, had they been available, would have detected variation between each of the five clones as well. Such markers were regrettably not available.

Concurrent collaboratives studies by Dr B.R.Shiels (Veterinary Parasitology, Glasgow) on the gene encoding the 30kDa merozoite surface protein of *T.annulata* (Dickson and Shiels, 1993) showed that the gene probe *TaA* 30 detected polymorphism between the five clones used in this study (Fig. 7.5). Analysis of other cloned cell lines (B.R.Shiels, personal communication), showed that the 8.3Kb and 6.4Kb fragments segregated i.e. cloned cell lines only exhibit either the 8.3Kb or the 6.4Kb fragment. This confirmed that clone 5 was composed of a mixture of two populations, as suspected from the data obtained with probe *TaT* 21 (Chapter 5, Section 5.3.4).

The third probe selected was probe *TaT* 21 which was already used to characterise the five clones as described in Chapter 5, Section 5.3.4.

Thus the infection of the calves with five populations, as defined by probe *TaT* 17, was in fact infection by six populations when also examined by probe *TaA* 30. However, analysis of the cell line established *in vitro* from the two batches of ticks fed on calves 320 and 360 by Southern blotting with the *TaA* 30 probe showed that only the 6.4Kb and 2.4Kb fragments were present. This suggests that the population identified by the 8.3Kb fragment did not

establish in the PBM (Fig. 7.6 B, track 1). This finding made the analysis of the clones derived from these cell lines, with probe *TaA* 30, more easily interpretable.

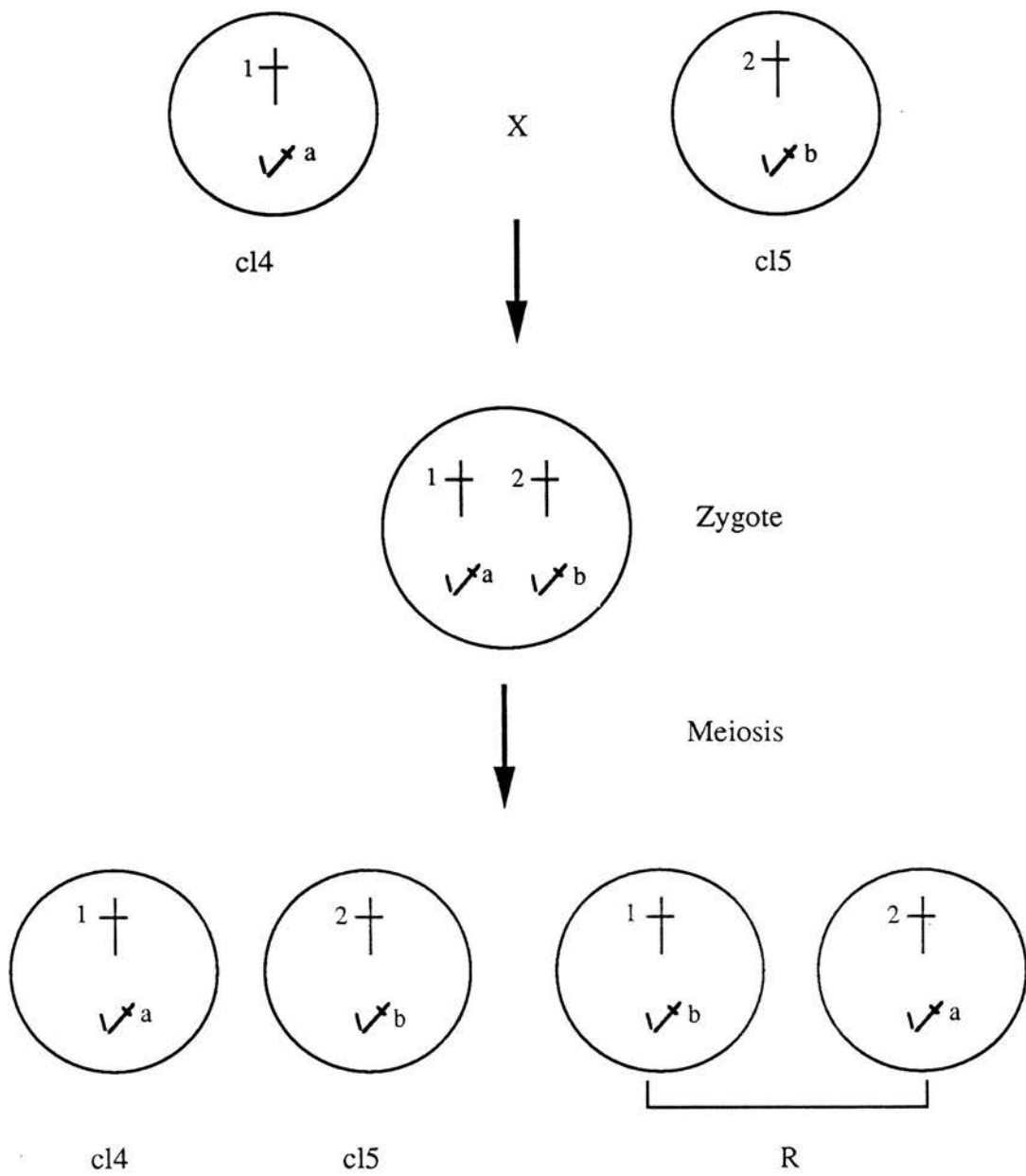
Indeed the results of analysis of clones derived from *TaTu* 320/260 showed that six out of ten presented novel genotypes when probed with *TaT* 17 and *TaA* 30. Since none of the cloned cell lines used to infect the calves had exhibited such profiles detected by these probes (Figs. 7.2 and 7.5), it was clear that these clones were recombinant and must have been generated by genetic exchange between the parental subpopulations.

The high proportion (six out of ten) of recombinant clones observed using these two markers suggested that they are unlinked i.e. located on different chromosomes or far apart on the same chromosome. It was established that *TaT* 17 and *TaA* 30 were located on different chromosomes (3 and 1 respectively), thus demonstrating that the loci defined by these probes are unlinked. This result would account for the high level of recombinants due to independent assortment of non-homologous chromosomes at meiosis as explained in Fig. 7.8.

Only one recombinant clone (out of nine) was detected in the progeny clones of cell line *TaT* 360/260, when analysed with probes *TaT* 21. The apparent low proportion of recombinant clones observed during the analysis suggests the loci defined by these probes are linked, i.e. located on the same chromosome. Indeed these probes were located on chromosome 3. This suggests that the recombinant clone was generated by crossing-over between homologous chromosomes. However, as explained by Fig. 7.9, some of the possible recombinants would not be detected during the analysis of the progeny of the cross, since they would have similar genotypes (according to probes *TaT* 17 and *TaT* 21), to the parental clones put into the animals i.e. clones 4 and 5. In these circumstances, the appreciation of the real proportion of recombinant cannot be detected and only an underestimation of the real events can be seen.

From the results discussed above, it could be concluded that *T.annulata* undergo a sexual phase in the tick vector as has been shown for *T.parva* (Morzaria *et al*, 1992b) and other protozoan parasites such as *Plasmodium* (Walliker, 1989). This sexual reproduction demonstrated by genetic exchange and recombination between different parasite subpopulations would contribute to the generation of diversity in *T.annulata* population.

**Figure 7.8** Recombinant clones obtained by independent assortment of non-homologous chromosomes



KEY:

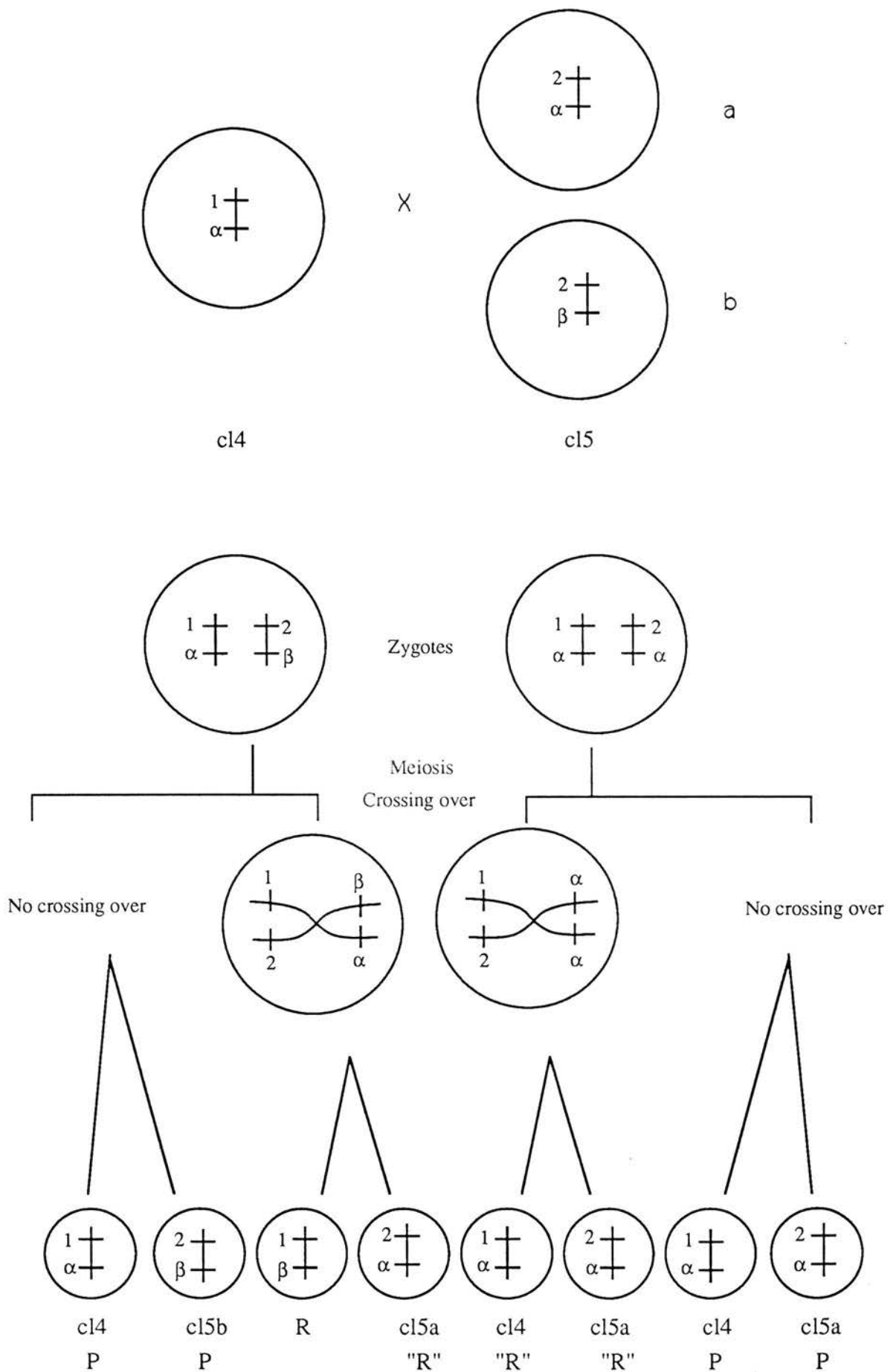
⊥ = chromosome 1 (*TaA30*)

✓ = chromosome 3 (*TaT17*)

R = recombinant



**Figure 7.9** Recombinant clones obtained by crossing-over between homologous chromosomes



**KEY:**

P= parental; R= Recombinant; 1,2= loci defined by probe *TaT17*; α,β= loci defined by probe *TaT21*.

## 7.5. IMPACT OF THE RESULTS ON VACCINATION

In this study, infectivity of five cloned *T.annulata*-infected lines, individually or as a mixture, for both vertebrate and invertebrate hosts was examined. Some of the findings need to be emphasised since they might have a repercussion on the use of cloned *T.annulata* infected cell lines, as vaccines, in the field.

The infections of the calves with cloned cell lines showed that four out of five clones were infective. The fifth clone, clone 2, did not infect calf 600 as assessed by detection of parasite or clinical and serological response. This was confirmed when the ticks fed on this calf did not show any sign of infection. Furthermore, Southern blot analysis of the cell lines derived from ticks fed on the calves inoculated with the pool of clones did not shown the presence of this clone either. Similarly this clone was not detected in the cell lines established from PBM of the same calves. No cell line could be established from calf 600 despite several attempts. All these data demonstrated that clone 2 was not infective to calves or had lost its infectivity after being cultivated *in vitro*.

This result is of some importance in that, when selecting vaccine candidates, some of these might behave like clone 2 from the start or at any time during their maintainance. It would be of interest to investigate the reasons behind this lack of infectivity to the bovine host and to determine whether it is related to a lack of transfer from the donor cells to the recipient cells or to an early destruction of the infected cells by non-specific immune response of the host.

The poor infectivity of the challenge made results difficult to interpret. However, on face value clone 5 and clone 1 gave better protection than clone 3 (compare PCV in Table 7.3). Clone 4 infection showed a greater level of pyrexia and so would be considered unsuitable as a vaccine candidate. In this context if a choice were to be made between clones 1 and 5 (and on the basis of these experiments only), then clone 1 would be a better vaccine candidate since it readily infects calves but was only transmitted to PBM by ticks in one occasion out of five i.e. has a low tick transmission index. This raises the issue of whether transmissibility of the vaccine parasite through ticks is required or not and if so to what extent. Targeting most of the cattle population, using a vaccine of clone 1 type would assume the

infection of the remaining unvaccinated animals, by tick transmission. A vaccine which is not transmissible by ticks might interfere with the stability of the disease in endemic areas in that, after the use of such vaccine for a number of seasons, the disease might be eradicated, the vaccination therefore stopped, and if disease was re-introduced, it could lead to a major outbreak since the animals would not be immune either due to the vaccination or natural challenge. If the vaccine is able to infect ticks and to be transmitted by these latter, then the endemicity is not disturbed and the use of such a vaccine would diminish the disease incidence while maintaining enzootic stability without eradicating the parasite.

This, however, has to be tested in the field and a number of conditions must be fulfilled before such a clone could be used as a vaccine. Satisfactory results in laboratory vaccination trials with a fully virulent challenge would be a first proviso, followed by cross-immunity tests using representative parasite strains, also in the laboratory. These would be precursors to the definitive evaluation of the candidate 'vaccine' at specific field sites.

The possibility that a parasite population used as a vaccine and transmissible through ticks, could recombine with other populations of *T.annulata* found in the field has to be considered since the experiments carried out in this chapter have proved that recombination can occur in *Hyalomma* ticks. The occurrence of recombination would imply the generation of new strains which might be of low or high virulence. This would need to be studied in more detail. If the vaccine strain recombines with natural populations of *T.annulata*, it would be diluted in the pool of existing parasites generating some of the novel strains. It is therefore unlikely that selection pressure would be exerted by the vaccine strain and lead to antigenic variants causing a breakthrough. If the vaccine strain does not recombine and its application is widespread, it might exert selection pressure which would produce populations against which the vaccine does not protect. However, investigations on parasite recombination in field conditions within *H.detrutum* have to be performed to establish the extent and implications of such recombination in natural populations.

## 7.6 CONCLUSION

The experiments described in this chapter analysed the biological characters exhibited by clones of *T.annulata* during infection and challenge of calves. They included the analysis of virulence and infectivity of well-defined cloned subpopulations, either individually or as a mixture, to both the vertebrate and arthropod host. Additionally, using genetic crosses in the ticks which were fed on calves infected with a mixture of clones, the question of whether a sexual phase, shown by recombination between clones, occurred in *T.annulata* was examined.

The immunisation of calves, with the same cell number of individual clones or a mixture of these, showed that overall the reactions observed in the second group were more severe than those observed in the first group. Although a large number of animals infected with individual clones needs to be examined, the results incline to an interpretation of potentiation and synergy between different subpopulations during mixed infections.

The challenge used to test the immunity provided by these clones was not fully virulent and, as such, less than satisfactory. It however, showed that some of the clones provided relatively better protection than others. In this preliminary study these results were primarily obtained in individual animals so the roles played by variation in individual response and the genomic type of these clones have still to be investigated by further experiments using larger groups of experimental animals.

In the experiments described in this section, it was shown that *T.annulata* clones could undergo genetic exchange and the results provide evidence of both independent assortment of non-homologous chromosomes and crossing-over between homologous chromosomes, generating recombinants that exhibit characters inherited from the two parental stocks. This is the first evidence for mating in *T.annulata* so far presented.

These experiments were carried out using a laboratory colony of *H.a.excavatum* ticks. This should not present major differences in parasite infection and transmission compared to the natural vector in Tunisia, *H.detrutum*, but this has to be ascertained by field investigations.

The experiment carried out answered some of the questions asked and gave indications of the possibilities for others as explained below.

1. The ability of individual cloned subpopulations to infect both vertebrate and invertebrate hosts is different from that of the pool of the same clones. This was shown by higher infection level in both animals and ticks when the mixture are used as compared to individual subpopulations. It was suggested that this might be due synergy and potentiation, although the precise basis of this phenomenon is unknown.
2. Individual clones proved to be infective for both calves and the ticks, but the level of infections varied from zero to levels similar to those obtained with the mixture of clones. This suggests that factors such as the animal or genotype of parasite may determine the infectivity of individual cloned populations.
3. As a result of the lack of infectivity/virulence of the parasite challenge, it is difficult to conclude anything about the ability of individual clones to induce protective immunity. This aspect has to be further examined in the future.
4. Although it could not be ascertained if cloned cell lines could be used as vaccines, it was conclusively shown that the clones have different abilities to infect calves. They are thus more than likely to have different abilities to protect if they are tested against a fully virulent challenge.
5. Whether attenuation is necessary to provide a vaccine has still to be determined. The clones used in this study might be found not to protect against a virulent challenge or might only protect for a short time compared to conventional attenuated vaccine.
6. The results showed that recombination does occur in *T.annulata*, generating a high level of diversity, according to the preliminary results. This does not exclude the possibility that clonality (Tibaryenc and Ayala, 1991) could occur as an alternative mode of reproduction of *T.annulata* in the field. However the high level of polymorphism detected in this study, with no two parasites being identical, would suggest that sexual reproduction occurs at a significant rate in the natural population.

In conclusion, it was shown that diversity in *T.annulata* might be generated through recombination within the vector tick. This phenomenon might be important for the parasite to escape the host's immune system by providing new recombinant genotypes different from the

existing ones and therefore assuring parasite survival. It was also shown that individual clones could potentially be used as vaccines rather than a mixture of them as the latter is responsible for marked clinical reactions during immunisation which cannot be accepted in vaccination trials. Accordingly when cell lines are attenuated in laboratories, to provide conventional vaccines, a clonal selection seems to occur. Indeed apparently most of the vaccines actually used are composed of individual homogeneous populations even though more than one genotype is detected in the original parental cell line at low passage (C.G.D.Brown, personal communication). Such cell lines are also usually too virulent to be used as vaccines.

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## *APPENDIX I*

*Giemsa stain*

#### 14. Preparation of Merck's Giemsa Stain

Reagents:	Glycerol (Analar)	540 ml
	Methyl alcohol (Solvent)	840 ml
	Merck's Giemsa powder	10 g
	Azur II	0.2 g/100 ml = 2.75 g

##### Method

1. Put Giemsa powder into large glass mortar. Add some of the glycerol and grind. Pour into large flat-bottomed flask.
2. Gradually wash with rest of glycerol into the flask.
3. Leave for 1 hour in 60°C water bath, shake intermittently.
4. Cool to room temperature.
5. Add 840 ml methanol, shake well.
6. Put on stirrer overnight.
7. Add Azur II.
8. Put on stirrer overnight or weekend.
9. Filter and store in a dark bottle (filter paper Whatmans No. 4).

##### Giemsa Buffer

Use buffer tablets GURR 65500  
1 tablet per litre of distilled deionised water - pH 7.2.

##### Staining slides with Giemsa Stain

1. Fix the prepared slides in technical methanol for 1-2 minutes.
2. Prepare solution of stain in Giemsa buffer\*.
3. Place slides in staining tray and cover with prepared Giemsa stain.
4. Leave for required length of time then rinse both sides of each slide with Giemsa buffer.
5. Wipe backs of slides with soft tissue and gently blot the fronts with filter or blotting paper.

\*The optimum effect is obtained by staining slides for 40 minutes in a 5% solution but slides may also be stained for 20 minutes at 10% or 10 minutes at 20%.

## *APPENDIX II*

### *Charts of calves*

*330*

*600*

*660*

*290*

*370*

*320*

*360*

*380*

*430*

*400*



# EXPERIMENTAL INFECTION

Title: Clonal Vaccine

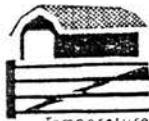
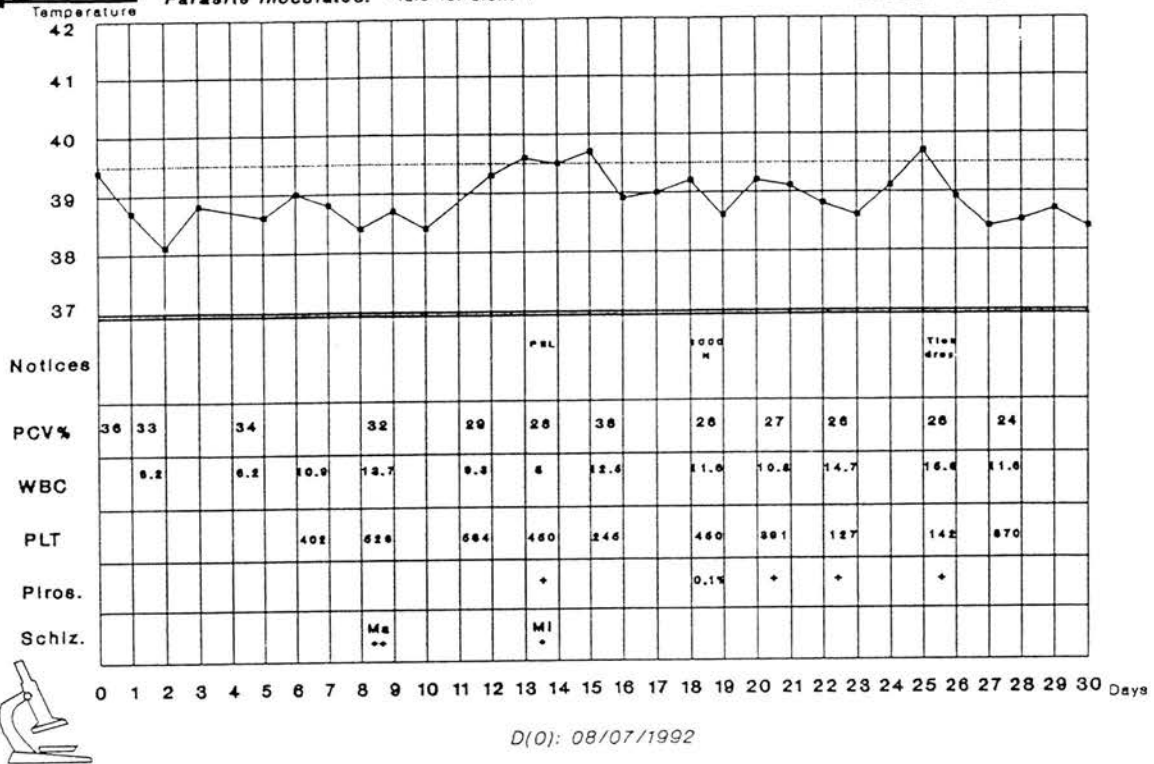
Calf number: 330

Number: 92/1A

Date of birth: 18/02/1992

Parasite inoculated: TaTu 13: clone 1

Breed: Frisonne x Holstein



# EXPERIMENTAL INFECTION

Title: Clonal Vaccine : Challenge

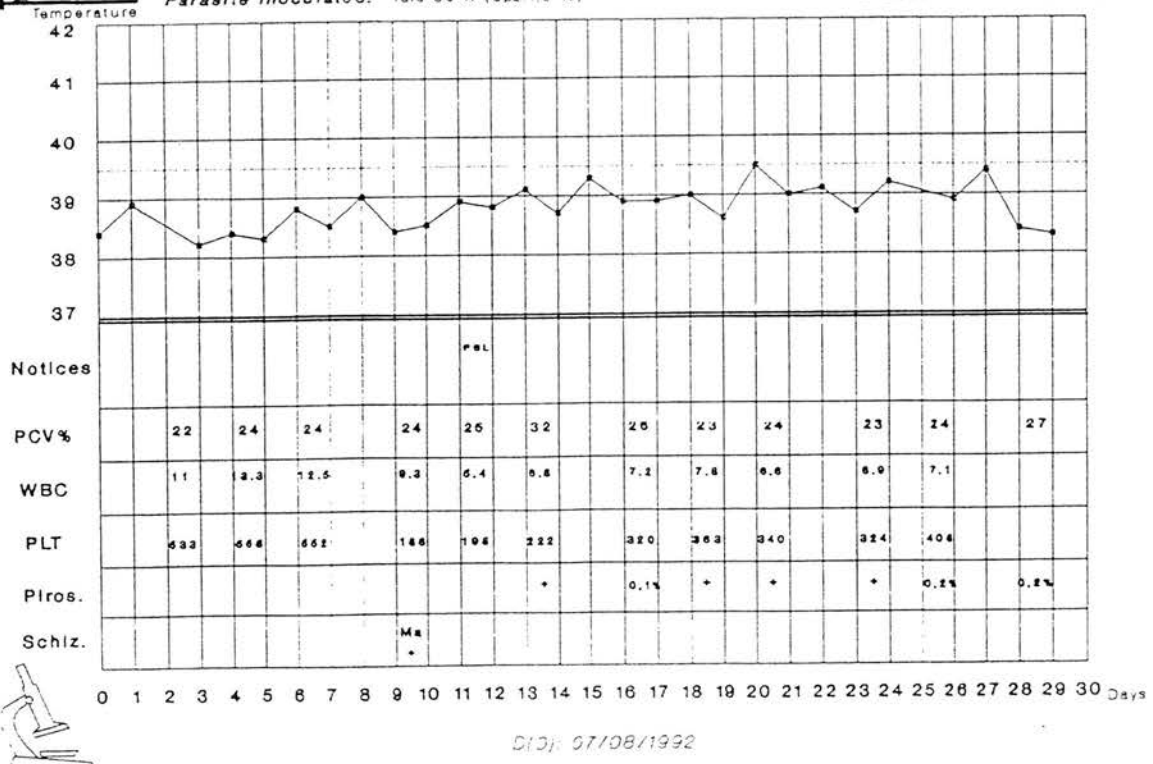
Calf number: 330

Number: 92/1B

Date of birth: 18/02/1992

Parasite inoculated: TaTu 09 A (Spz: 1.6 te)

Breed: Frisonne x Holstein





# EXPERIMENTAL INFECTION

Title: Clonal Vaccine

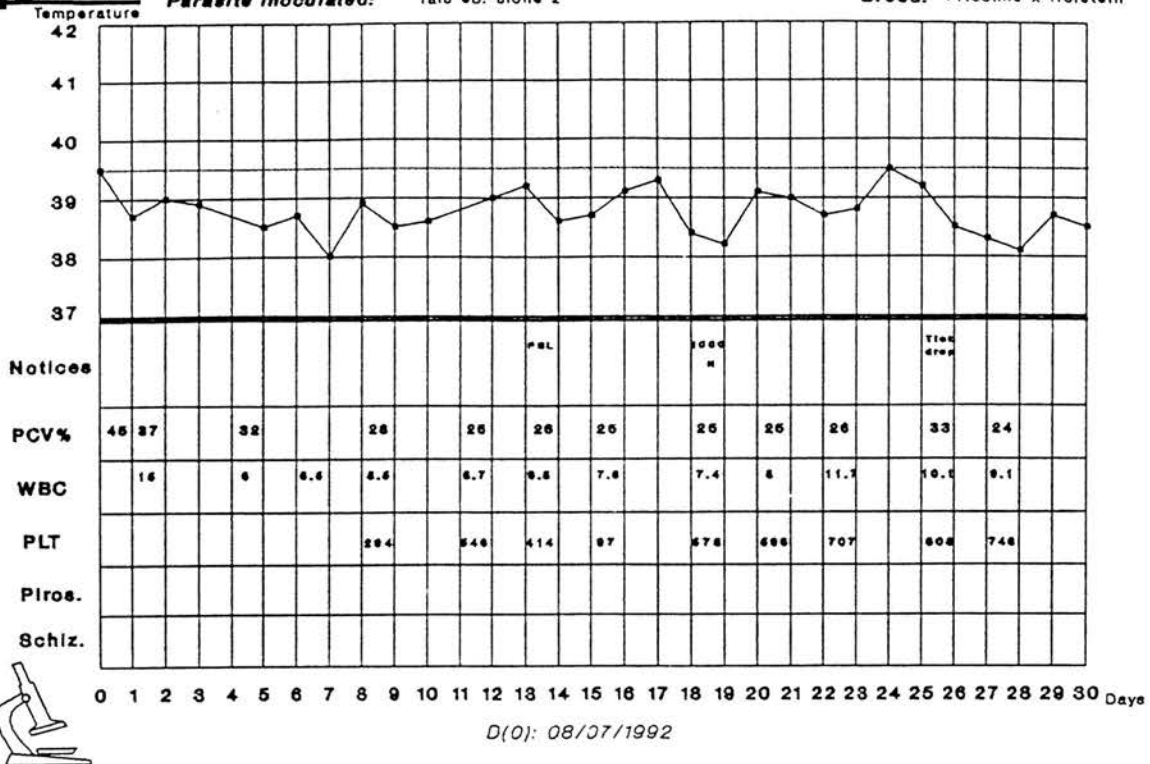
Calf number: 60Q

Number: 92/1A

Date of birth: 11/03/1992

Parasite inoculated: TaTu 9B: alone 2

Breed: Frisonne x Holstein



# EXPERIMENTAL INFECTION

Title: Clonal Vaccine - Challenge

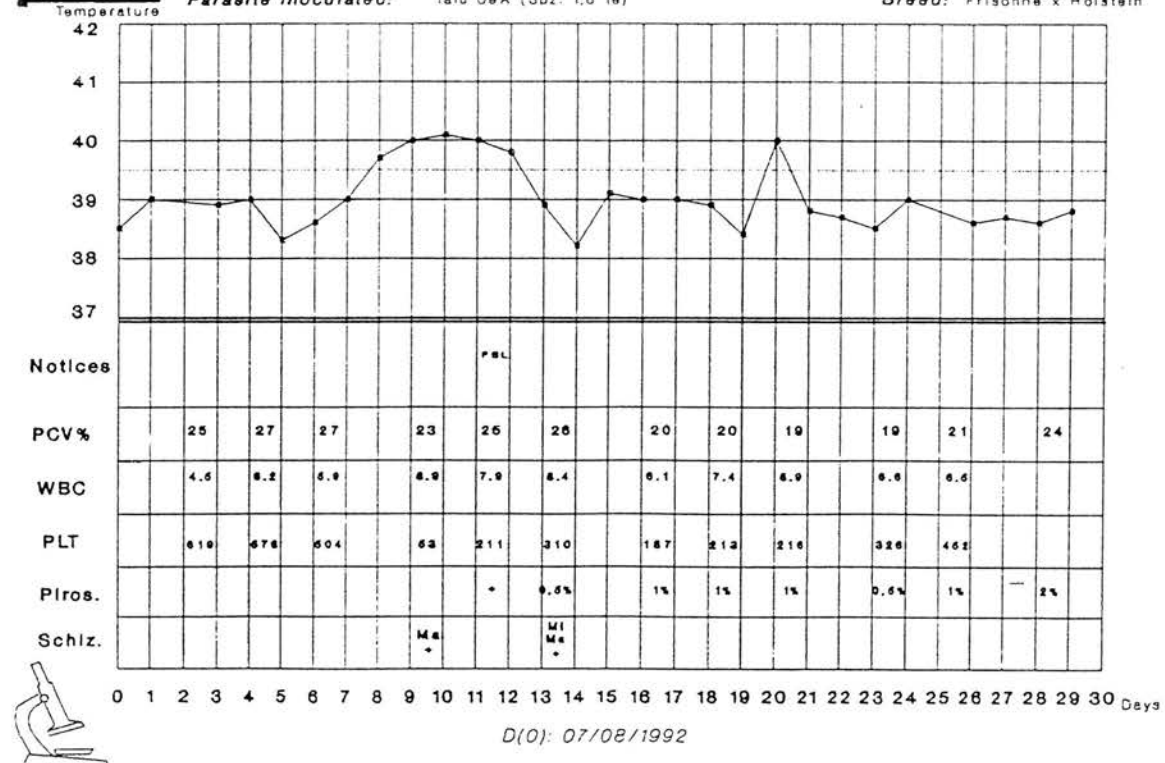
Calf number: 60C

Number: 92/1B

Date of birth: 11/03/1992

Parasite inoculated: TaTu 09A (Spz: 1.6 te)

Breed: Frisonne x Holstein

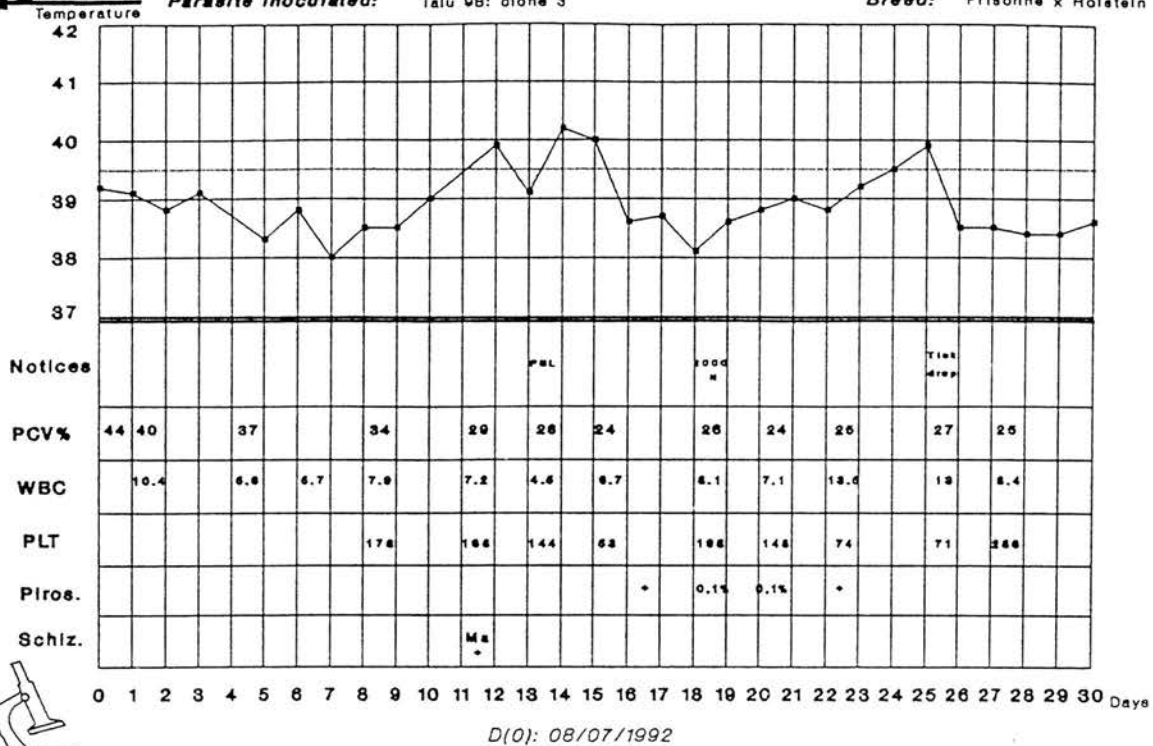




# EXPERIMENTAL INFECTION

Title: Clonal Vaccine  
Number: 92/1A  
Parasite inoculated: TaTu 98: clone 3

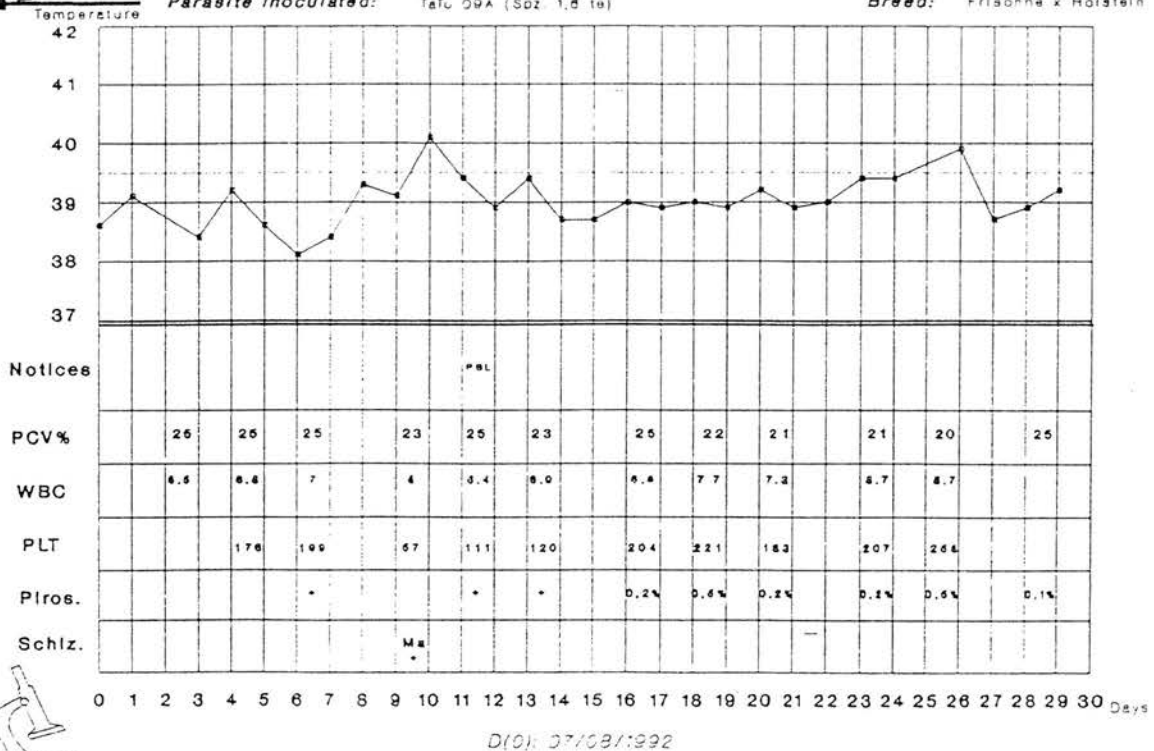
Calf number: 660  
Date of birth: 22/03/1992  
Breed: Frisonne x Holstein



# EXPERIMENTAL INFECTION

Title: Clonal Vaccine Challenge  
Number: 92/1B  
Parasite inoculated: TaTu 99A (Spz. 1.5 te)

Calf number: 660  
Date of birth: 22/03/1992  
Breed: Frisonne x Holstein

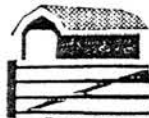
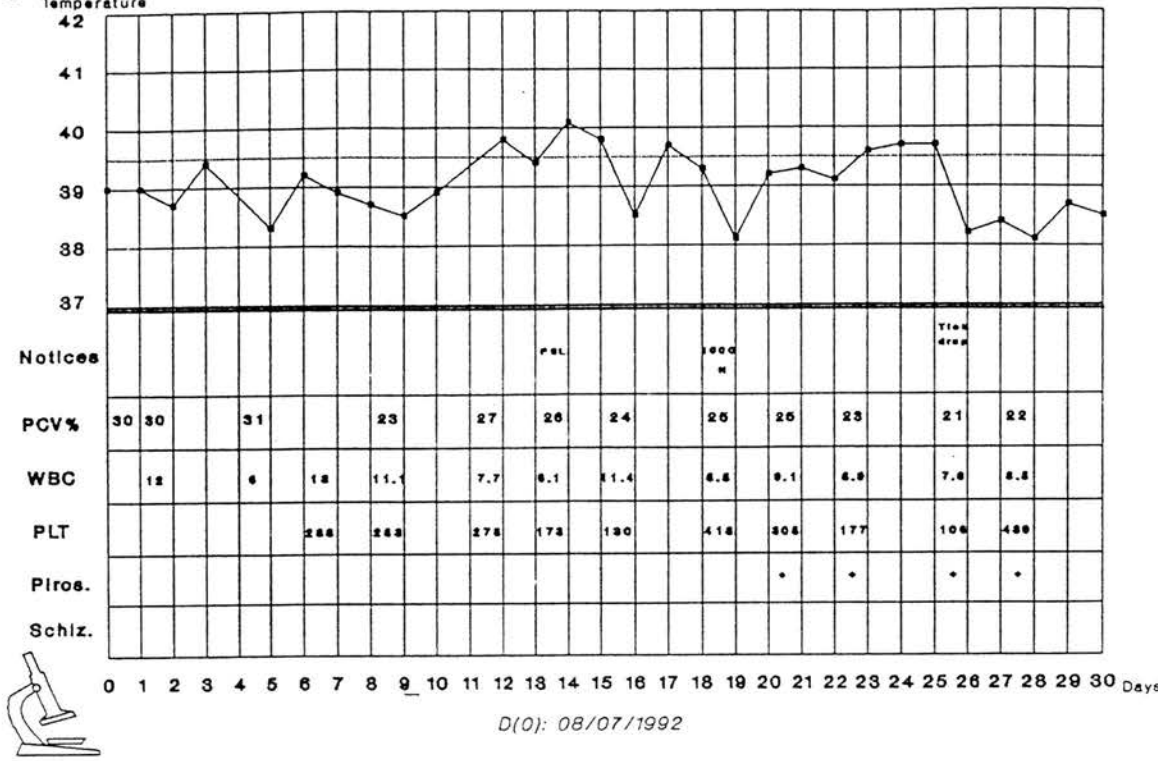




# EXPERIMENTAL INFECTION

Title: Clonal Vaccine  
Number: 92/1A  
Parasite inoculated: TaTu 9A: clone 4

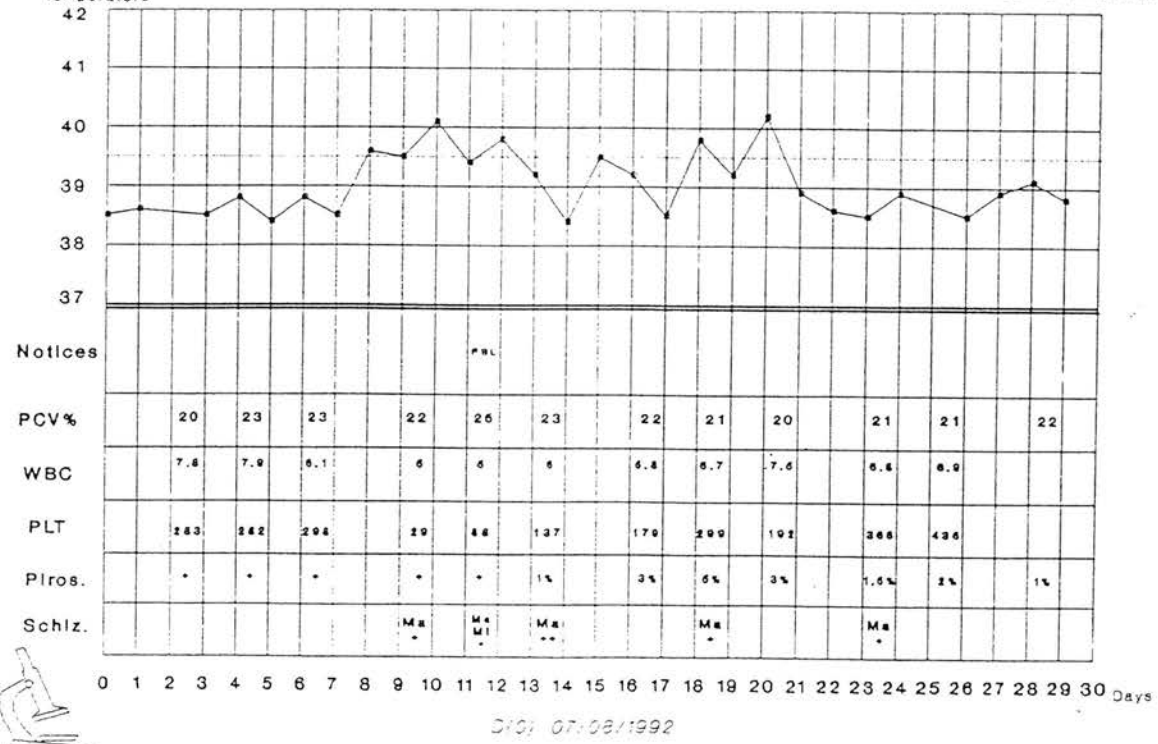
Calf number: 200  
Date of birth: 15/02/1992  
Breed: Frisonne x Holstein



# EXPERIMENTAL INFECTION

Title: Clonal Vaccine Challenge  
Number: 92/1B  
Parasite inoculated: TaTu 09A (Sp2: 1.6 1e)

Calf number: 290  
Date of birth: 15/02/1992  
Breed: Frisonne x Holstein







# EXPERIMENTAL INFECTION

Title: Clonal Vaccine

Calf number: 370

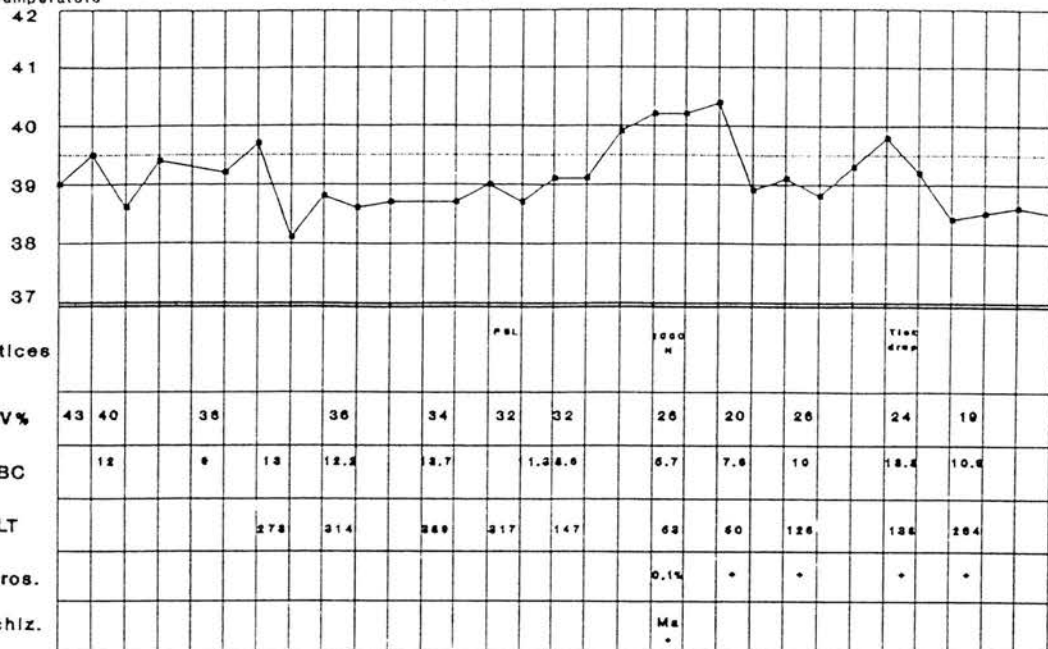
Number: 92/1A

Date of birth: 29/02/1992

Parasite inoculated: TaTu 02: clone 5

Breed: Frisonne x Holstein

Temperature



Notices

PCV%

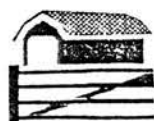
WBC

PLT

Piros.

Schiz.

D(0): 08/07/1992



# EXPERIMENTAL INFECTION

Title: Clonal Vaccine Challenge

Calf number: 370

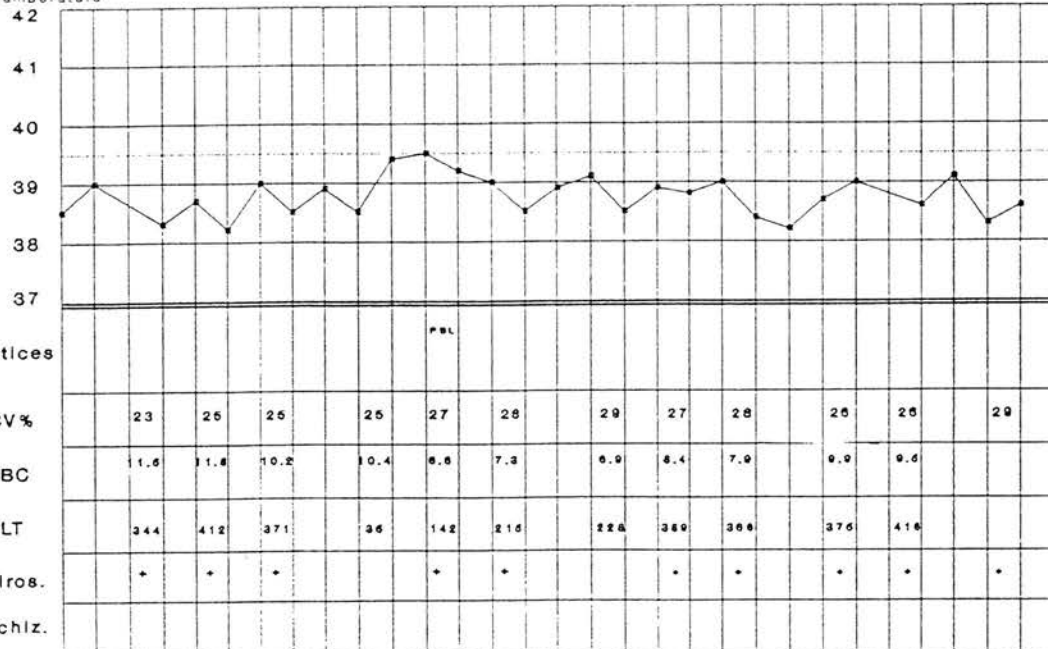
Number: 92/1B

Date of birth: 29/02/1992

Parasite inoculated: TaTu 09A (Spz : 1.6 te)

Breed: Frisonne x Holstein

Temperature



Notices

PCV%

WBC

PLT

Piros.

Schiz.

D(0): 07/08/1992





## EXPERIMENTAL INFECTION

Title: Clonal Vaccine

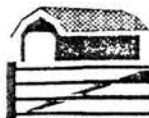
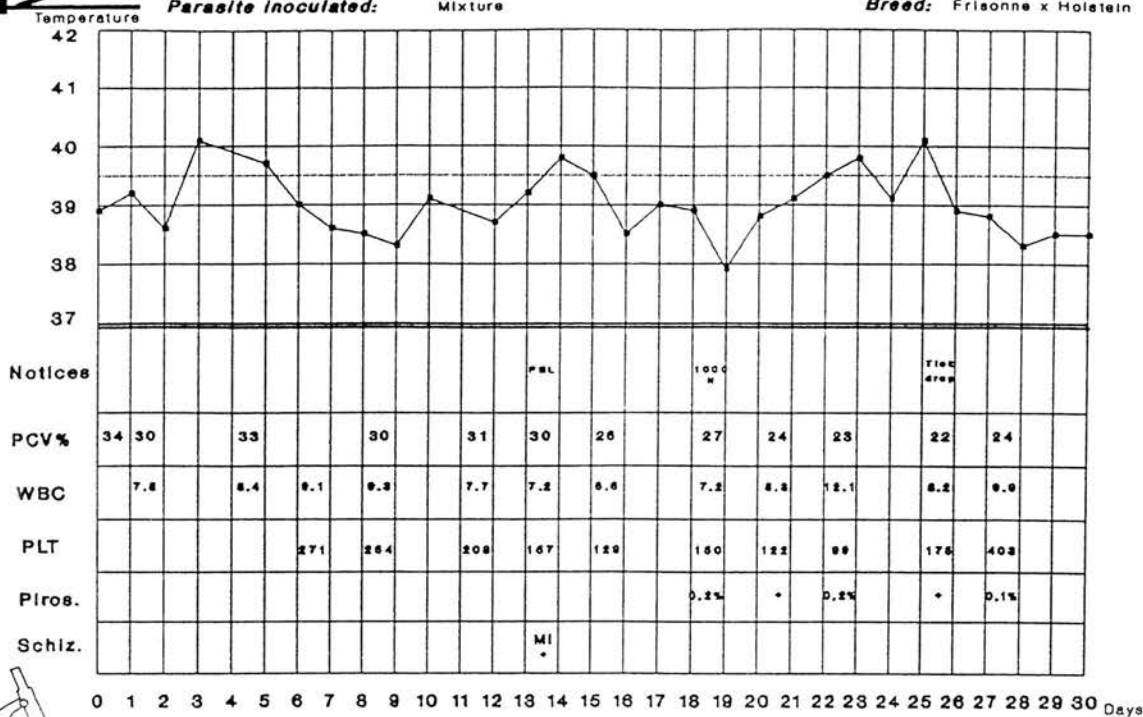
Calf number: 320

Number: 02/1A

Date of birth: 17/02/1992

Parasite inoculated: Mixture

Breed: Frisonne x Holstein



## EXPERIMENTAL INFECTION

Title: Clonal Vaccine : Challenge

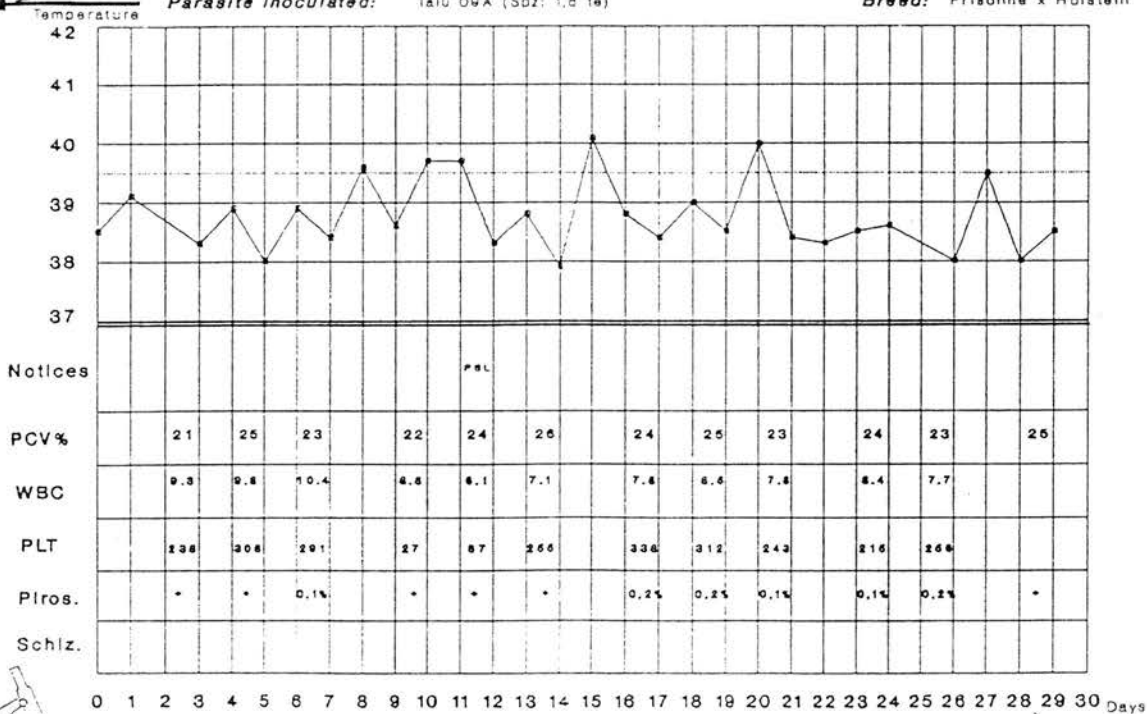
Calf number: 320

Number: 02/1B

Date of birth: 17/02/1992

Parasite inoculated: TaTu 09A (Spz: 1.0 1e)

Breed: Frisonne x Holstein

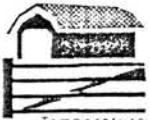
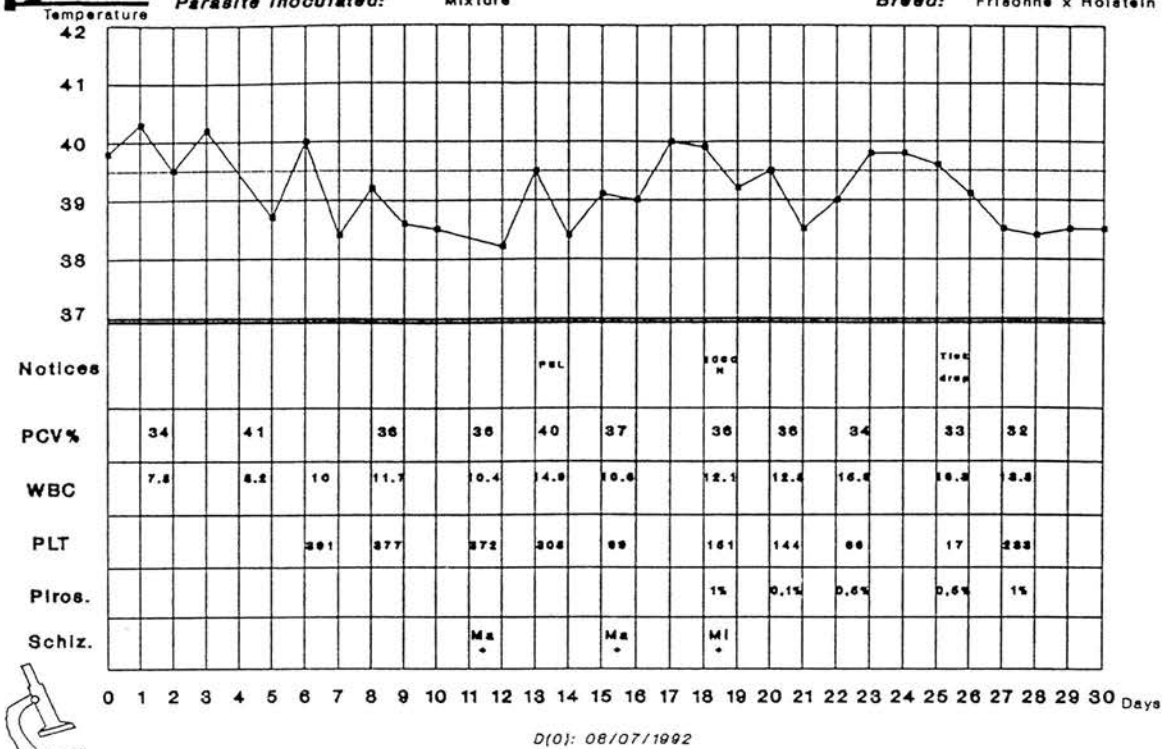




# EXPERIMENTAL INFECTION

Title: Clonal Vaccine  
Number: 92/1A  
Parasite inoculated: Mixture

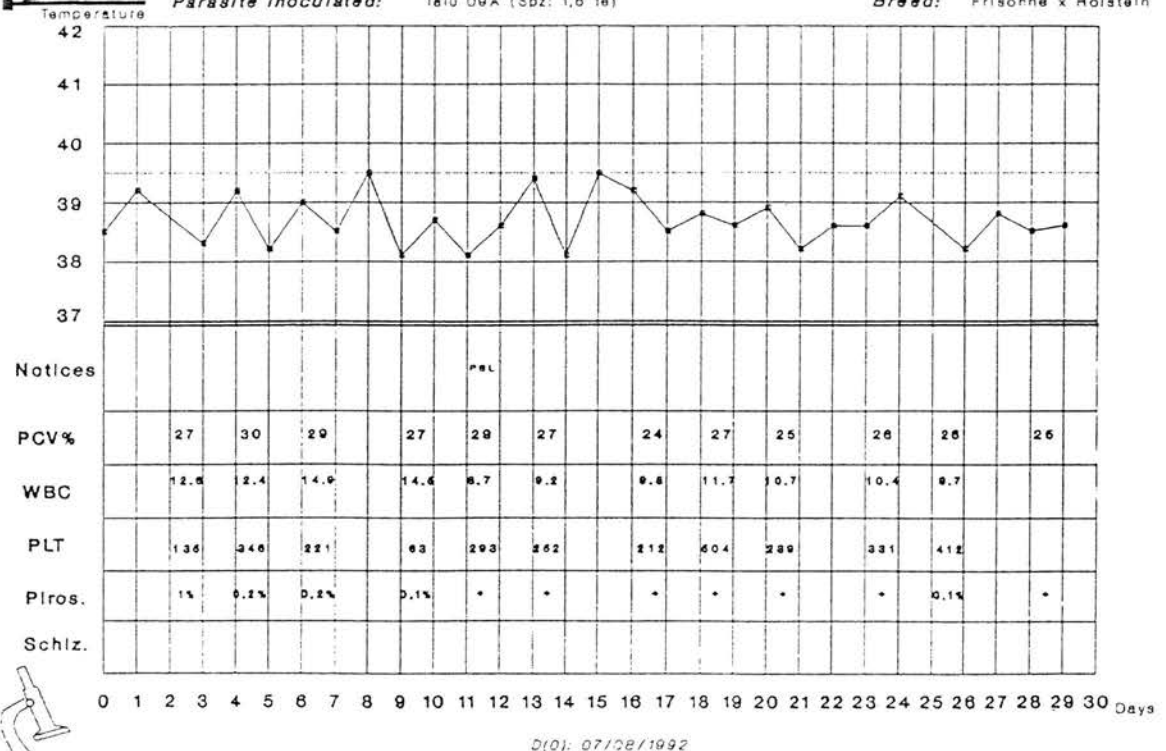
Calf number: 360  
Date of birth: 27/02/1992  
Breed: Frisonne x Holstein



# EXPERIMENTAL INFECTION

Title: Clonal Vaccine : Challenge  
Number: 92/1B  
Parasite inoculated: TeTu 09A (Spz: 1,6 1e)

Calf number: 360  
Date of birth: 27/02/1992  
Breed: Frisonne x Holstein

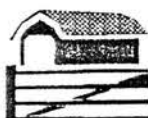
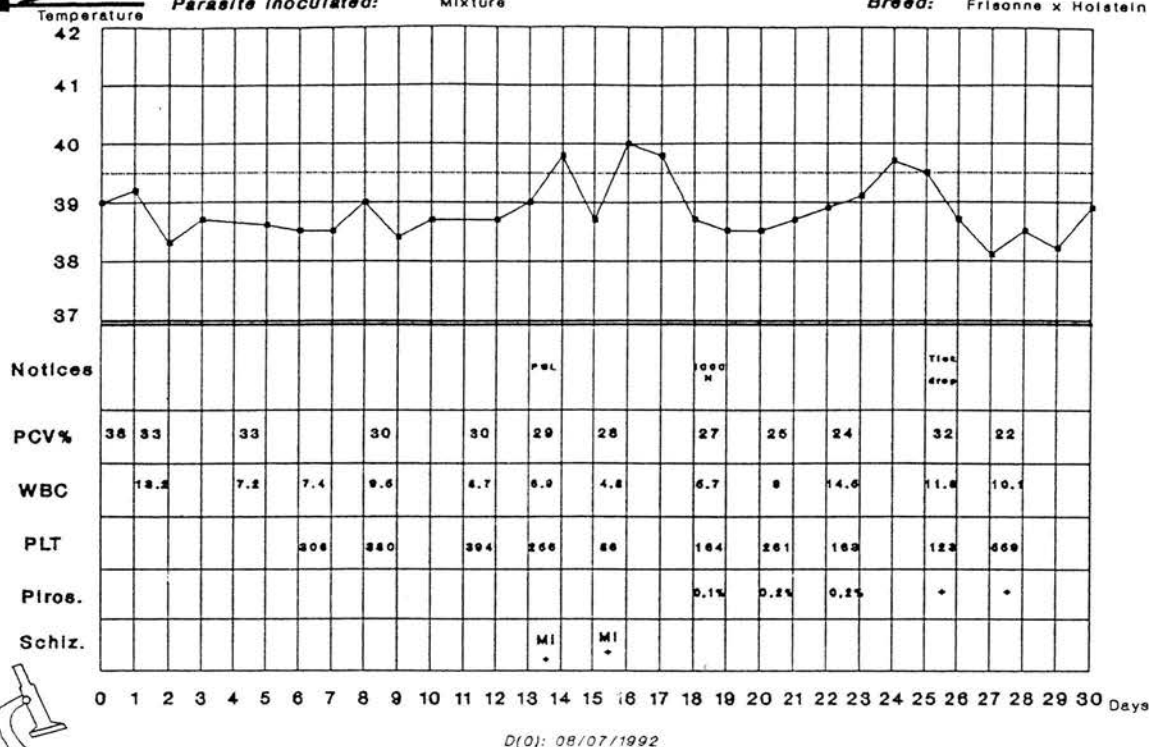




## EXPERIMENTAL INFECTION

Title: Clonal Vaccine  
Number: 92/1A  
Parasite inoculated: Mixture

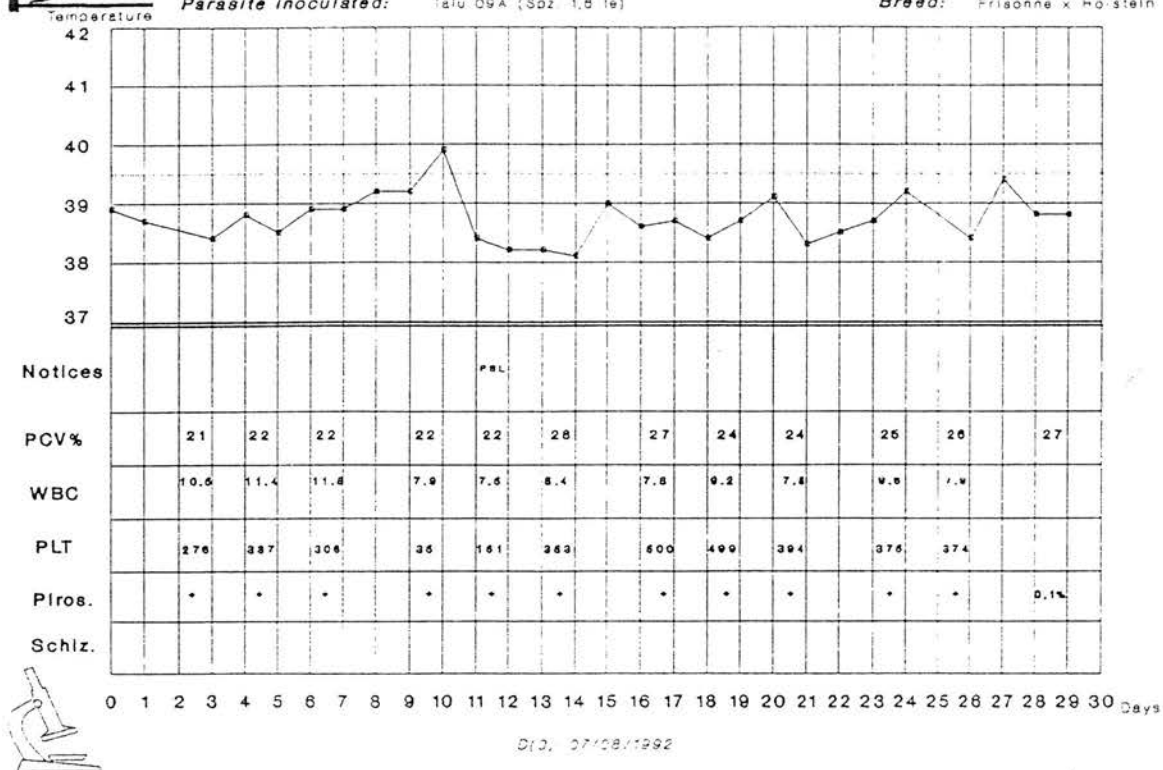
Calf number: 380  
Date of birth: 28/02/1992  
Breed: Frisonne x Holstein



## EXPERIMENTAL INFECTION

Title: Clonal Vaccine Challenge  
Number: 92/1B  
Parasite inoculated: TaTu 09A (Spz: 1,5 lg)

Calf number: 380  
Date of birth: 28/02/1992  
Breed: Frisonne x Holstein



## EXPERIMENTAL INFECTION

**Title:** Clonal Vaccine

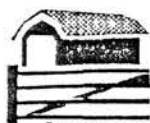
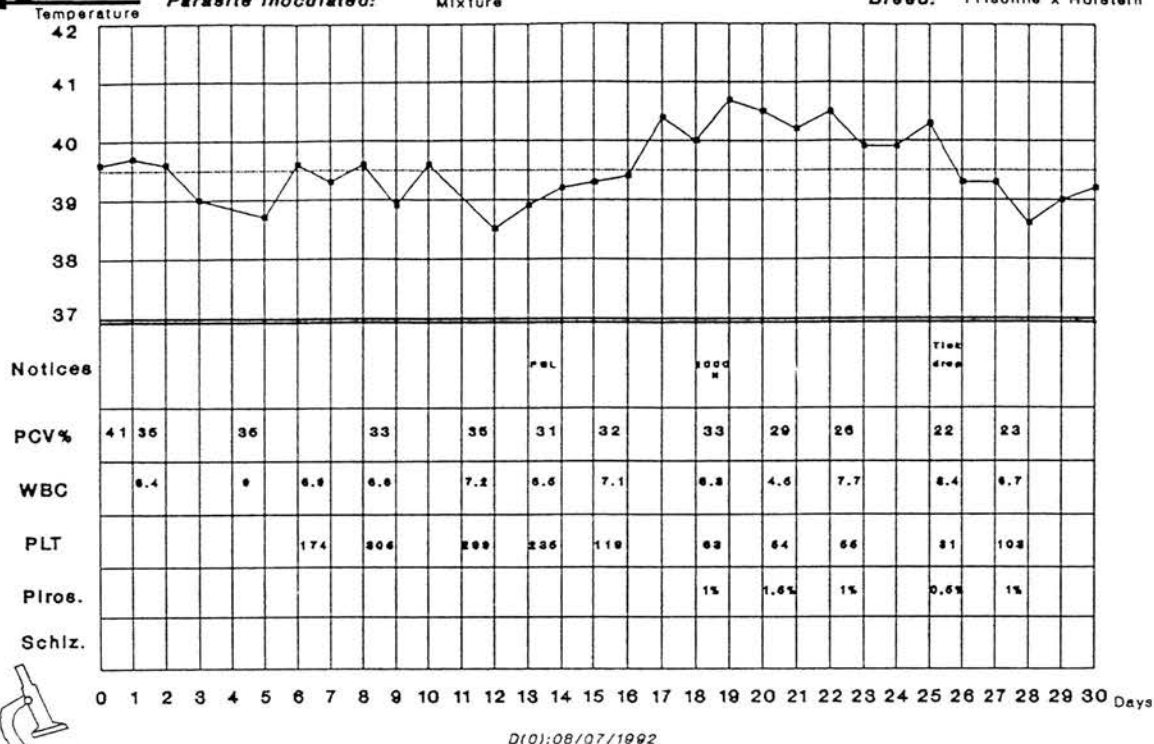
Calf number: 430

Number: 02/1A

**Date of birth:** 10/03/1992

Parasite Inoculated: Mixture

**Breed:** Friaonne x Holstein



## EXPERIMENTAL INFECTION

**Title:** Clonal Vaccine : Challenge

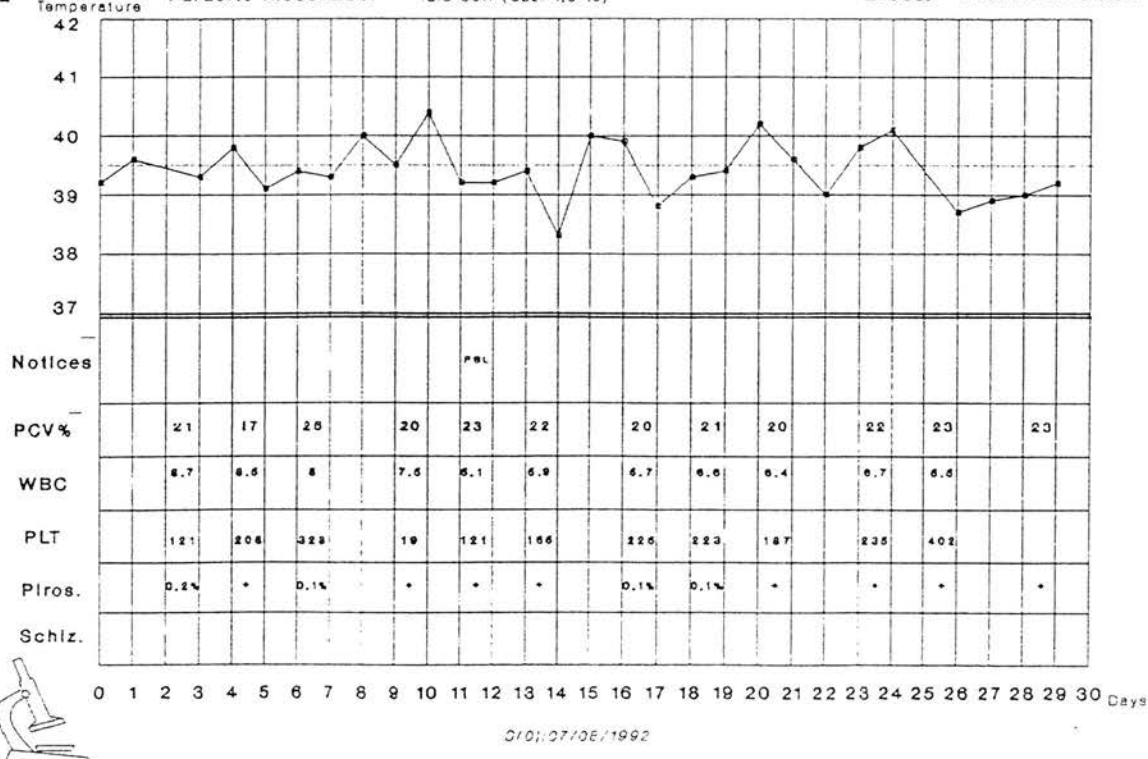
Calf number: 430

Number: 92/18

Date of birth: 10/03/1992

*Parasite inoculated:* TaTu 09A (Spz: 1.6 te)

**Breed:** Frisonne x Holstein





## EXPERIMENTAL INFECTION

Title: Piroplasm selection in vivo

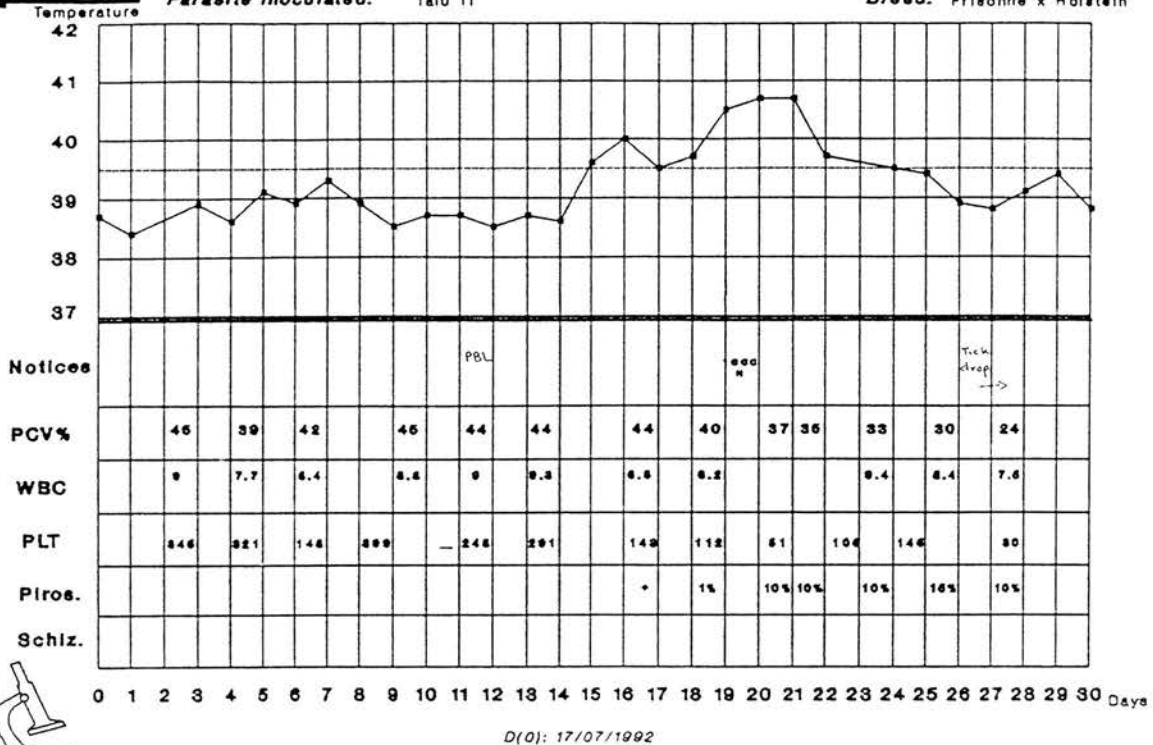
Calf number: 400

Number: 92/2

Date of birth: 07/03/1992

Parasite inoculated: TaTu 11

Breed: Frisonne x Holstein



## EXPERIMENTAL INFECTION

Title: Piroplasm selection in vivo

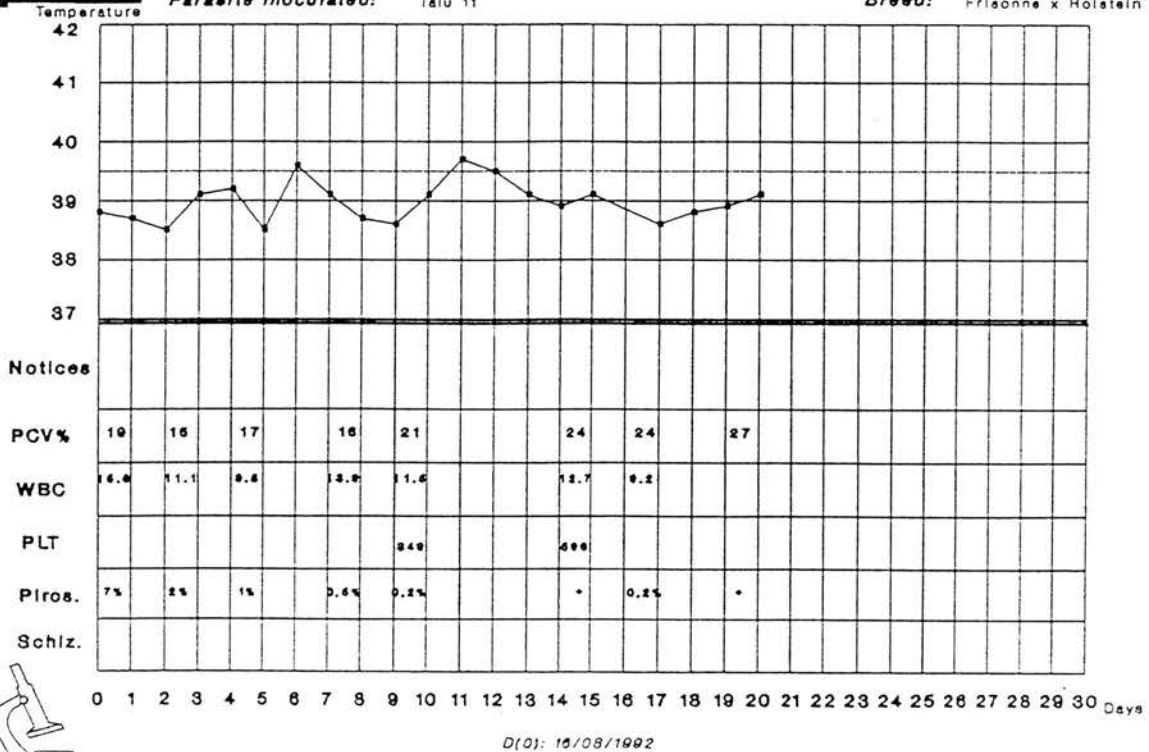
Calf number: 400

Number: 92/2

Date of birth: 07/03/1992

Parasite inoculated: TaTu 11

Breed: Frisonne x Holstein



## *PUBLICATIONS*

**The following publications included work contained in this thesis:**

1. Disruption of synchrony between parasite growth and host cell division is determinant of differentiation to the merozoite in *Theileria annulata*. (1992) Brian Shiels, Jane Kinnaird, Susan McKellar, Joanne Dickson, Leila Ben Miled, Ron Melrose, Duncan Brown and Andy Tait. *Journal of Cell Science*, 101, 99-107.
2. Genomic and phenotypic diversity of Tunisian *Theileria annulata* isolates. (1994) L.Ben Miled, K.Dellagi, G.Bernardi, T.R.Melrose, M.Darghouth, A.Bouattour, J.Kinnaird, B.Shiels, A.Tait and C.G.D.Brown. *Parasitology*, 108 (in press).



## Disruption of synchrony between parasite growth and host cell division is a determinant of differentiation to the merozoite in *Theileria annulata*

BRIAN SHIELS<sup>1,\*</sup>, JANE KINNAIRD<sup>1</sup>, SUSAN McKELLAR<sup>1</sup>, JOANNE DICKSON<sup>1</sup>, LEILA BEN MILED<sup>2</sup>, RON MELROSE<sup>2</sup>, DUNCAN BROWN<sup>2</sup> and ANDY TAIT<sup>1</sup>

<sup>1</sup>Wellcome Unit of Molecular Parasitology, Department of Veterinary Parasitology, University of Glasgow, Glasgow G61 1QH, Scotland, UK

<sup>2</sup>Centre for Tropical Veterinary Medicine, University of Edinburgh, Roslin, Scotland, UK

\*Author for correspondence

### Summary

The multinucleated macroschizont stage of the protozoon *Theileria annulata* is an intracellular parasite of bovine leukocytes. The parasite induces the host cell to proliferate, and divides in synchrony with the immortalised host cell. Differentiation to the next stage occurs within the host cell culminating in the release of merozoites and destruction of the leukocyte. In this study clones of *Theileria annulata* macroschizont-infected cell lines were isolated by limiting dilution and tested for differentiation to the merozoite stage (merogony). Two cloned cell lines underwent differentiation with enhanced efficiency, while two others were of lower efficiency. Quantification was carried out using monoclonal antibodies, which showed that over 90% of the cells in an enhanced cloned cell line could be induced to differentiate. By carrying out induction at 41°C for limited periods of time followed by culture at 37°C

evidence was obtained that differentiation to the merozoite is a two-step process: a preliminary reversible phase, followed by a second irreversible phase of differentiation. Analysis of the nuclear number of the macroschizont and the growth rate of the cloned cell lines showed that the ability to differentiate was associated with an increase in nuclear number (size) of the macroschizont, generated by a disruption in the synchrony between parasite growth and host cell division. We believe that these results reveal a relationship between a reduction in parasite division and differentiation, and that there are similarities between stage differentiation in parasites and cellular differentiation in higher eukaryotes.

Key words: *Theileria*, differentiation, cell division.

### Introduction

Differentiation of a cell frequently occurs after changes in the cellular environment. In higher eukaryotes these changes can be caused by an alteration in the level of polypeptide growth factors, which regulate the proliferative activity of cellular populations (Cross and Dexter, 1991). Differentiation of protozoan parasites from one life-cycle stage to another can also occur in response to environmental changes, particularly during the transition from the invertebrate vector to the vertebrate host. The molecular mechanisms that regulate parasite stage differentiation have not been fully elucidated, although it has been postulated that an increase in heat shock gene expression is involved (Van Der Ploeg et al., 1985).

*Theileria annulata* is a protozoon parasite of cattle and is the causative agent of tropical theileriosis, a disease of livestock in areas of Europe, North Africa and Asia (Purnell, 1978). The bovine phase of the life

cycle (Fig. 1) is initiated by inoculation of the sporozoite stage with a feeding tick. The sporozoite invades mononuclear leukocytes and differentiates through the trophozoite to the macroschizont stage (Jura et al., 1983). Macroschizont-infected host cells become immortalised, and division of parasite and host cell occurs in synchrony (Hulliger, 1965), the parasite associating closely with the microtubules of the host cell spindle to achieve distribution of the schizont to the daughter leukocytes. Differentiation to the merozoite stage (merogony) takes place within the host cell with the generation of merozoite nuclei, rhoptry and microneme organelles (Melhorn and Schein, 1984). Differentiation culminates in the destruction of the leukocyte and the release of merozoites into the extracellular environment. Free merozoites subsequently invade erythrocytes, where they mature into piroplasms (Conrad et al., 1985). Completion of the cycle occurs with the uptake of infected erythrocytes by the tick vector.

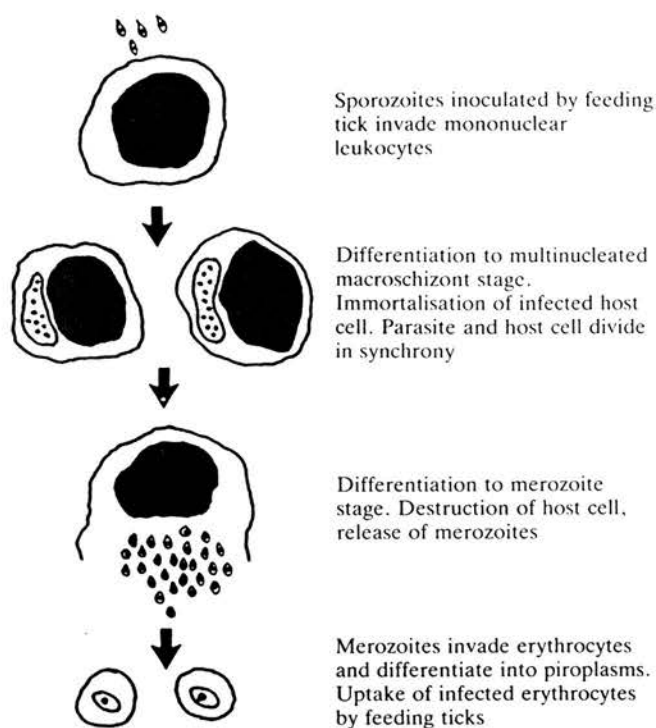


Fig. 1. Schematic outline of *T. annulata* stage differentiation in the bovine host.

In a previous study differentiation of the macroschizont to the merozoite was induced by raising the temperature of the culture to 41°C. Reactivity of macroschizont-infected cells and merozoites with a panel of monoclonal antibodies demonstrated that this process results in significant changes in the antigenic profile of the parasite, indicating that merogony is a major point of differentiation in the mammalian phase of the parasite life cycle and that both positive and negative regulation of gene expression occur during this process (Glascodine et al., 1990). These experiments were carried out with a macroschizont-infected cell line derived by infection of peripheral blood mononuclear cells with sporozoites from a parasite stock. It has been shown that parasite stocks, and the macroschizont cell lines derived from them, consist of more than one type of parasite, which can be distinguished by monoclonal antibody reactivity and restriction fragment length polymorphisms on Southern blots (Shiels et al., 1986; Conrad et al., 1989; Toye et al., 1991). In the present study we have isolated clones of macroschizont-infected cell lines by limiting dilution, and demonstrated that certain cloned cell lines undergo differentiation with a high degree of efficiency. Using these clones we have obtained evidence that merogony is a two-step process, with a preliminary reversible phase leading to a second irreversible phase of differentiation. In addition, we present data that demonstrate that a disruption in the synchrony between parasite growth and host cell division is a determinant of differentiation to the merozoite, and that similarities exist between differentiation in parasites and higher eukaryotic cells.

## Materials and methods

### Cell culture and cloning of the TaA2 macroschizont-infected cell line

The *T. annulata* (Ankara) macroschizont-infected cell line (TaA2) was obtained by *in vitro* infection of peripheral blood mononuclear cells with sporozoites from a mixed parasite stock. Maintenance of the cell line in culture was carried out at 37°C in RPMI-1640 (Gibco) supplemented with 20% heat-inactivated foetal calf serum, 8 µg ml<sup>-1</sup> streptomycin, 8 units ml<sup>-1</sup> penicillin, 0.6 µg ml<sup>-1</sup> amphotericin B and 0.05% NaHCO<sub>3</sub>. Cloning of a low passage number of this cell line was carried out by limiting dilution as previously described (Shiels et al., 1986). To determine whether the cell lines had isoenzyme patterns characteristic of clones, analysis was performed for glucose 6-phosphate isomerase as outlined by Melrose et al. (1984).

### Induction of differentiation in vitro

Induction of macroschizont-infected cell lines to differentiate was carried out by increasing the temperature of culture from 37°C to 41°C. The cell number of each culture was estimated by counting, using a haemocytometer, and was adjusted to 1.4 × 10<sup>5</sup> cells ml<sup>-1</sup> by dilution with fresh medium, before and every second day after transfer to 41°C.

### Giemsa staining and indirect immunofluorescence assay

Morphological examination of induced cultures was routinely carried out by light microscopy of Giemsa-stained cytospin preparations. A 50 µl sample of culture was spun at 1,500 revs min<sup>-1</sup> (240 g) for 5 min using a Shandon cytospin 2. The preparations were then air dried for 10 min and fixed in methanol for 30 min. Staining was performed with Gurr's improved R66 Giemsa stain (BDH) at 4% in distilled water.

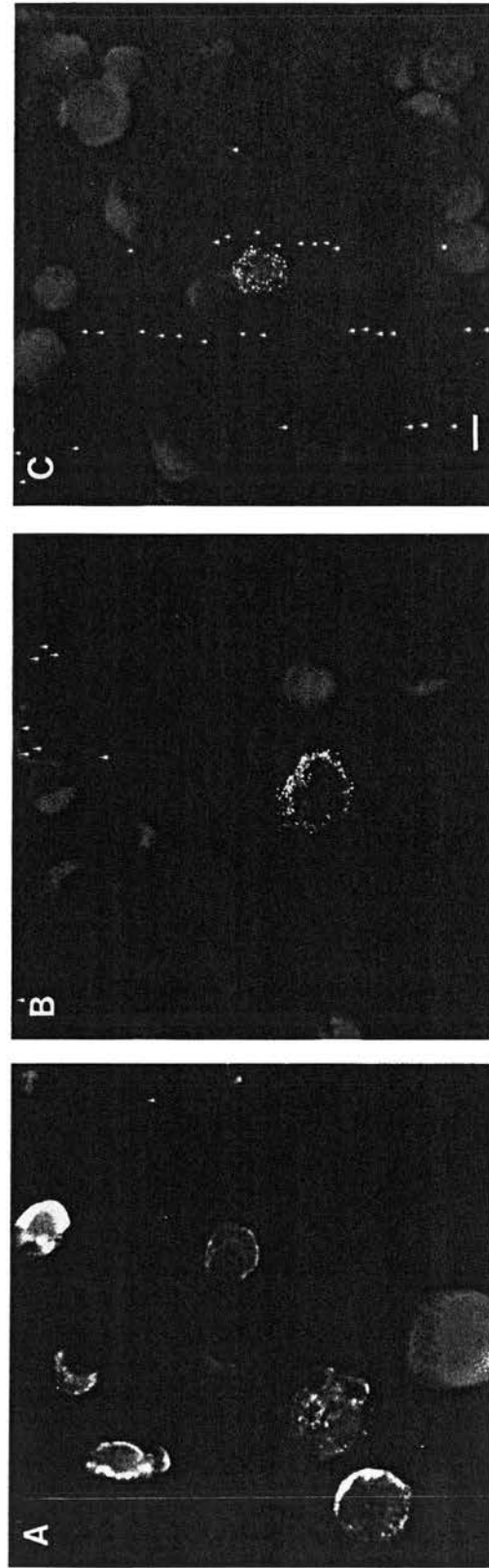
Indirect immunofluorescence assay (IFA) on preparations of differentiating cultures with monoclonal antibodies raised against the piroplasm stage of the parasite (Glascodine et al., 1990) was carried out as described previously (Shiels et al., 1986). Differentiating cultures were centrifuged at 500 g for 5 min and washed three times with sterile phosphate-buffered saline (PBS). Fixation was carried out on ice in 1.8% formaldehyde (BDH) for 10 min and the cells were then washed three times in PBS. Cells were resuspended in PBS (approx. 5 × 10<sup>6</sup> ml<sup>-1</sup>), spotted onto PTFE Multispot slides (C.A. Hendley, Essex) and air dried. Analysis and photography of immunofluorescence was carried out with a Leitz Ortholux II fluorescent microscope and an Orthomat-W camera attachment.

### Southern blotting

The isolation of DNA from the different cell lines, gel electrophoresis and Southern blotting were performed using standard methods (Maniatis et al., 1982). The L16 DNA used as a probe was isolated from a genomic library of *T. annulata* (Jedaida 2), cloned in pUC 18. The probe was radiolabelled by the random priming method (Feinberg and Vogelstein, 1983). Hybridisation and washing were carried out at 65°C as described by Church and Gilbert (1984).

### Counts and statistical analysis

To quantify the number of cells that reacted with the monoclonal antibodies by IFA the numbers of positive and negative cells were counted in random microscope fields. Between 500 and 1000 cells were counted for each time point and cell line. Statistical analysis of the values obtained for the



**Fig. 3.** Immunofluorescence reactivity of monoclonal antibodies 5E1, 1C2 and 1D11 against fixed slide preparations of cloned cell lines C9 and E3 after 8 days of culture at 41°C. (A) Reactivity of cloned cell line C9 with monoclonal 5E1; (B) reactivity of cloned cell line E3 with monoclonal 1C2; and (C) reactivity of

cloned cell line E3 with monoclonal 1D11. Note the peripheral labelling with monoclonal antibody 5E1, and the distinct stippled fluorescence with monoclonal antibodies 1C2 and 1D11. Bar, 10  $\mu$ m.

different cell lines was carried out using the  $\chi^2$  distribution and  $2 \times 2$  contingency tables. Estimation of the macroschizont nuclei number was carried out by counting the nuclei of over 100 infected cells in random fields of Giemsa-stained cytospin preparations. This was done for both cloned cell lines at each time point. The standard error of the mean (S.E.M.) was estimated, and the difference between means tested, using standard statistical formulae (Harper, 1971). In order to analyse the growth rate of the different cell lines the cell number per ml was estimated by counting samples in a haemocytometer, after the culture had been diluted to approximately  $1.4 \times 10^5$  cells  $\text{ml}^{-1}$  at the end of a period of growth. After a further 48 h of culture the cell number was again estimated, and the increase in cell number was calculated by dividing the second count by the first. This procedure was carried out at  $37^\circ\text{C}$  and at 2-day intervals at  $41^\circ\text{C}$ , until day 6. Counting was carried out in duplicate for duplicate cultures of each cloned cell line. The S.E.M. for each time point was estimated, and the difference between means tested by using the  $t$  distribution.

## Results

### Isolation of cloned macroschizont-infected cell lines

Four cell lines were successfully isolated by limiting dilution of the TaA2-infected macroschizont cell line and showed clonal phenotypes by isoenzyme analysis (data not shown). These cloned cell lines were then tested for their ability to differentiate at  $41^\circ\text{C}$ . Two of these clones (C9 and D7) exhibited morphology characteristic of differentiating cultures after 10 days at  $41^\circ\text{C}$  (Fig. 2B and C). In approximately 50% of these cells the number of nuclei was greatly increased and the parasite was enlarged, filling most of the host cell cytoplasm. In just over one third of these parasites the nuclei were bulky and stained densely. The macroschizont was so large in some cases that it completely filled the host cell, which also appeared to be enlarged. In addition to the enlarged macroschizonts, parasites containing multiple small densely staining particles were observed within approximately 22% of the host cells. This morphology is indicative of merozoite formation, and from previous studies these forms were identified as microschizonts. A high level of extracellular particles (between 300 and 400 per microscope field) with small densely staining nuclei were also observed, and these were identified as merozoites (Glascodine et al., 1990).

After 10 days at  $41^\circ\text{C}$  the level of differentiation of the other two cloned cell lines (E3 and D3) did not appear as extensive (Fig. 2F). Only 8% of the infected host cells contained enlarged macroschizonts (volume of parasite greater than 50% of the host cell cytoplasm), with approximately 1% showing the microschizont morphology consistent with merozoite formation. The small extracellular densely staining merozoites were observed at low levels (20–30 per field). Continued culture over 14 days at  $41^\circ\text{C}$  resulted in the loss of the cultures of cloned cell lines C9 and D7. In contrast, after a period of poor growth (from day 4 to 14) the cloned cell lines D3 and E3 recovered and could be kept at  $41^\circ\text{C}$  indefinitely. These cells showed the morphology

of macroschizont-infected cells (see Fig. 2H), with an occasional infected cell displaying morphology consistent with merozoite formation. From these initial observations we designated the cloned cell lines having an enhanced (C9 and D7) or a diminished (E3 and D3) differentiation phenotype.

### Reactivity of differentiating parasites with monoclonal antibodies

Monoclonal antibodies have been raised that react against the merozoite and piroplasm, but not against the macroschizont of *T. annulata* (Glascodine et al., 1990). Using IFA, three of these monoclonals (5E1, 1D11 and 1C2) were tested against fixed slide preparations of the cloned cell lines after 8 days of culture at  $41^\circ\text{C}$ . Fig. 3A shows that within the represented field the majority (but not all) of cells of the enhanced clone cell line (C9) reacted with monoclonal antibody 5E1, with most of the fluorescence being located at the periphery of the infected cell. None of the cells of the diminished cloned lines (D3 or E3), however, showed reactivity with antibody 5E1 (Table 1).

In contrast to 5E1, antibody 1C2 clearly reacted against a small proportion of cells of the diminished (D3 and E3) cloned cell lines but showed no reactivity against cells of the enhanced (D7 or C9) cloned cell lines. The staining pattern differed from that of monoclonal 5E1 in that the fluorescence obtained was stippled and distinct (see Fig. 3B). The third monoclonal (1D11) gave the same staining pattern as monoclonal 1C2 (Fig. 3C) but, unlike the other two antibodies, reacted against cells of all the cloned cell lines (Table 1). From these results we concluded that in addition to reacting with the liberated merozoite the three antibodies also detected parasites that were in the process of merogony, and could therefore be used as markers for differentiation.

### Genotypic analysis of cloned cell lines by Southern blotting

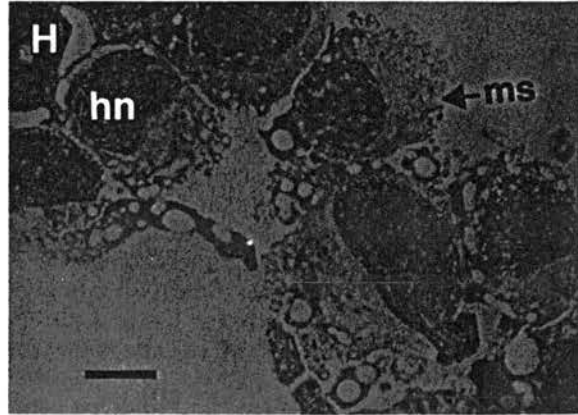
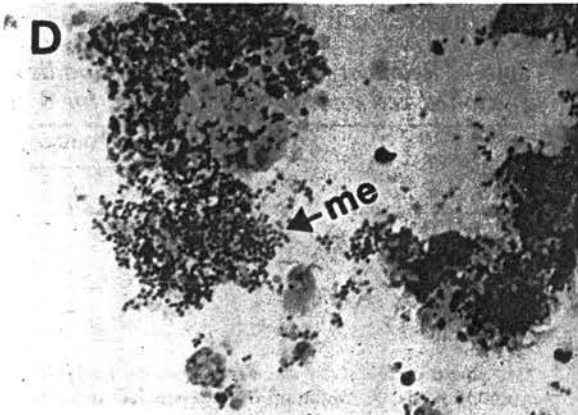
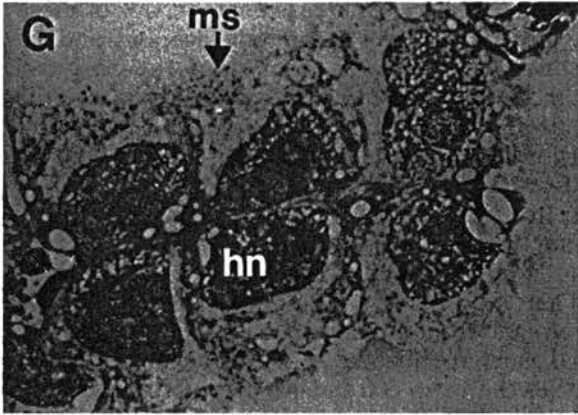
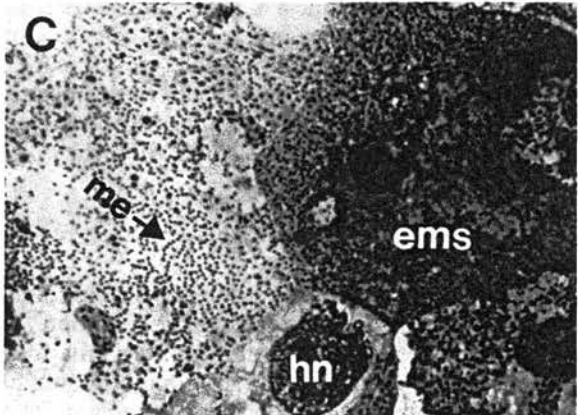
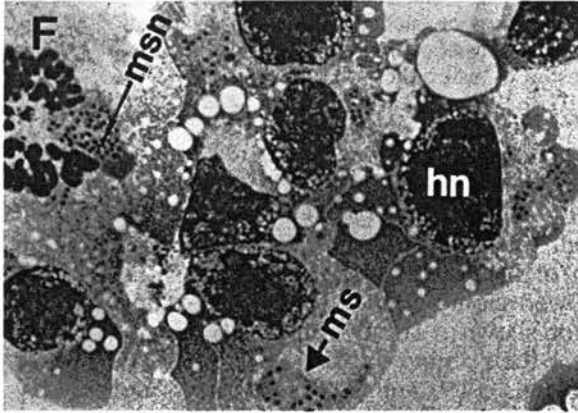
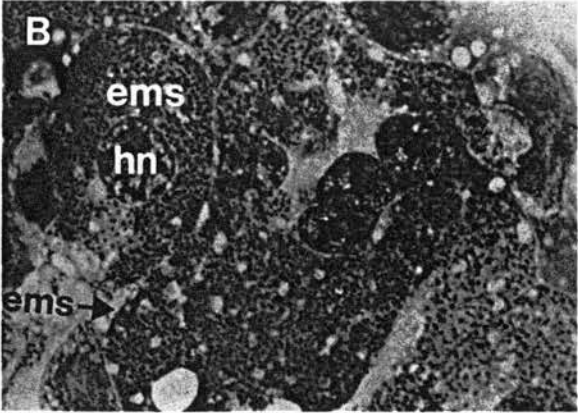
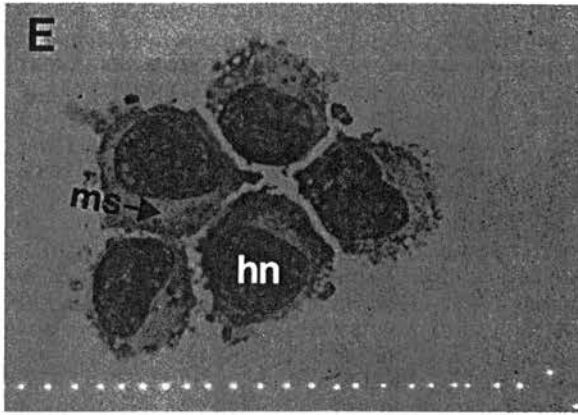
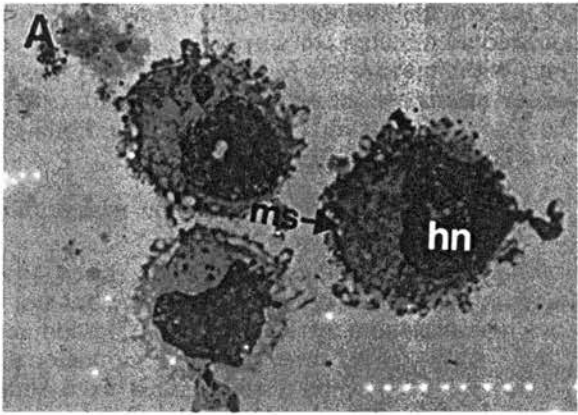
In order to determine whether any differences between the enhanced and the diminished cloned cell lines existed at the genomic level, Southern blot analysis was carried out. A restriction fragment length polymorphism was observed when the *T. annulata* DNA probe L16 (Ben Miled, unpublished data) was hybridized to *Eco*RI-digested cell line DNA. This was most evident

**Table 1.** Reactivity of monoclonal antibodies against cloned cell lines cultured at  $41^\circ\text{C}$  for 8 days

	Monoclonal antibody		
	5E1	1C2	1D11
Cell line D7	+	–	+
C9	+	–	+
E3	–	+	+
D3	–	+	+
TaA2	+	+	+

Assessment of reactivity was carried out by analysis of random microscope fields. A minimum of between 200 and 500 cells were analysed for each antibody and cell line.





**Fig. 2.** Giemsa-stained cytocentrifuge preparation of *T. annulata* (Ankara) cloned cell lines C9 and E3 following culture at 41°C. (A-D) Cloned cell line C9. (A) At 37°C; (B) day 6 at 41°C; (C) day 10 at 41°C; and (D) day 16 at 41°C. (E-H) Cloned cell line E3. (E) At 37°C; (F) day 6 at 41°C; (G) day 10 at 41°C; and (H) day 44 at 41°C. Host cell nucleus (hn), macroschizont (ms), macroschizont nuclei (msn), enlarged macroschizont (ems) and merozoites (me). Bar, 10 µm.

in the major bands of 7.1 kb and 1.3 kb, which were specific to either the enhanced (7.1 kb; Fig. 4, tracks 2 and 3) or the diminished (1.3 kb; Fig. 4, tracks 4 and 5) cloned cell lines. Both of these bands were present in DNA isolated from the *T. annulata* (Ankara) cell line TaA46 (Fig. 4, track 1).

#### Quantification of differentiation using monoclonal antibodies 1D11 and 5E1

To compare the ability of enhanced (cloned cell line C9), diminished (cloned cell line E3) and parental (TaA2) cell lines to differentiate, fixed preparations of cells after different time points of culture at 41°C were tested by IFA with monoclonal antibodies 1D11 and 5E1, and the percentage of positive cells was estimated. The analysis with monoclonal 1D11 (Table 2) showed that at all time points tested the percentage of cells that reacted positively to antibody 1D11 was highest with the enhanced cloned cell line (C9). At day 12 the percentage of positive cells for this cell line was nine times greater than that estimated for the diminished cloned cell line (E3) and over seven times greater than the parental cell line (TaA2). The number of positive cells in the diminished cloned cell line (E3) increased to 8% at day 14 (data not shown), but after culture for 44 days at 41°C this level was reduced to 0.2%.

Owing to the inability of monoclonal antibody 5E1 to detect differentiating cells of the diminished cloned cell lines (see Table 1) the analysis with this antibody was confined to the enhanced cloned cell line (C9) and the parental line (TaA2). At day 10 over 80% of the cells of clone C9 reacted with monoclonal 5E1, and this high level increased to 92% at day 12. These values compared with a maximum (at day 10) of 18% for the parental cell line (TaA2).

In addition to comparing the abilities of the three cell lines to differentiate, the quantitative analysis showed a difference in the number of cells of the enhanced cloned



**Fig. 4.** Southern blot analysis of *EcoRI* restriction-digested DNA isolated from different cell lines. The blot was hybridised with the *T. annulata* L16 probe as described in Materials and methods. Track 1, DNA isolated from cell line TaA46; tracks 2 and 3, DNA isolated from cloned cell lines C9 and D7; tracks 4 and 5, DNA isolated from cloned cell lines E3 and D3.

cell line (C9) that reacted with antibody 5E1 compared with antibody 1D11. Thus, after 10 days of culture at 41°C three times more cells were positive with antibody 5E1 than with 1D11. Interestingly, by day 12 the difference was reduced to one and a half times.

#### Limited incubation of cultures at 41°C results in a reduction in the level of differentiation

To test whether placing the cultures of macroschizont-infected cells at 41°C resulted in a rapid induction of differentiation, the enhanced cloned cell line (C9) was placed in culture at 41°C. Following incubation for different time periods at the increased temperature, cultures were returned to 37°C. The cultures were then maintained at 37°C up to the time at which visible differentiation, as assessed by Giemsa-stained morphology, had occurred in the control culture at 41°C. Fixed slide preparations were then made and the level of differentiation was assessed by IFA with monoclonal 5E1. Table 3 shows that 24 h at 41°C followed by 6 days at 37°C produced the same result as the negative control (37°C) culture in that no cells were induced to differentiate by this procedure. A proportion of the cells (14%) were positive for differentiation after 48 h in

**Table 2.** Percentage of cells positive with monoclonal 5E1 or 1D11 before and after induction at 41°C

Days at 41°C	0		10		12		44
Monoclonal antibody	5E1	1D11	5E1	1D11	5E1	1D11	1D11
Cell line C9	0	0	82	26	92	63	n.d.
E3	0	0	n.d.	2	n.d.	7	0.2
TaA2	0	0	18	8	5	9	n.d.
			*	†	*	†	

n.d., test not done, due to non-reactivity of monoclonal antibody 5E1 or the inability of cell line C9 to survive culture at 41°C.

\*Significant difference with monoclonal antibody 5E1 between cloned cell line C9 and cell line TaA2 ( $P<0.01$ ).

†Significant difference with monoclonal antibody 1D11 between cloned cell lines C9 and E3, and between C9 and TaA2 ( $P<0.01$ ).

**Table 3.** Percentage cells positive with monoclonal 5E1 after different times of culture at 41°C, followed by culture at 37°C

Days at 41°C	Days at 37°C	% Cells 5E1 +ve $\pm$ S.D.
0	7	0
1	6	0
2	5	14 $\pm$ 1.4
3	4	30 $\pm$ 5.6
4	3	50 $\pm$ 2.1
7	0	73 $\pm$ 2.1

Values are the mean obtained from duplicate cultures  $\pm$  the standard deviation (S.D.). The duplicate values at each time point were compared by  $\chi^2$  analysis. The only time point to show a significant difference between the duplicate cultures was day 3 at 41°C ( $P<0.01$ ).

culture at 41°C followed by 5 days at 37°C. This proportion increased the longer the cells were maintained at the higher temperature, so that after 4 days at 41°C followed by 3 days at 37°C 50% of the cells were estimated to be differentiating. In agreement with the above observation, it was found that the highest level of differentiation (73%) was achieved by continuous culture at 41°C for 7 days (see Table 3).

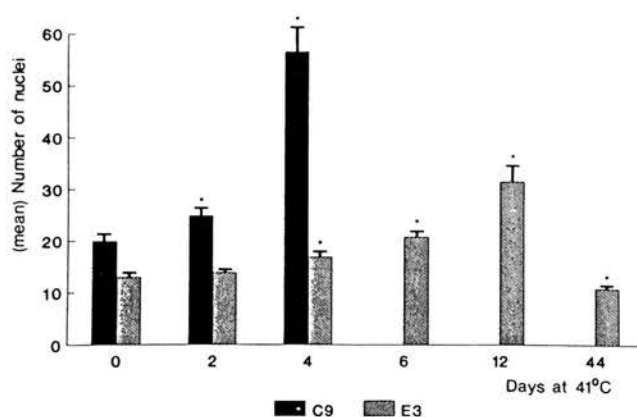
#### Estimation of number of macroschizont nuclei during culture at 41°C

Morphological analysis of macroschizont-infected cells cultured at 41°C has shown an increase in the number of nuclear particles per macroschizont, which is associated with a larger parasite size, during differentiation to the merozoite (Hulliger et al., 1966; Glascodine et al., 1990; see Fig. 2B). To compare the extent of these changes in the enhanced (C9) with those in the diminished (E3) cloned cell lines, the mean number of nuclei per macroschizont was estimated at successive 2-day time points at 41°C, and the data are presented in Fig. 5. For the enhanced cloned cell line (C9) at 37°C the average number of nuclei per schizont was 20. This number increased by 40% (8 nuclei) after 2 days of culture at 41°C, and over the next 48 h between day 2 and day 4 the mean number of nuclei rose from 27 to 57, an increase of almost 300% from day 0. An accurate estimation of the number of nuclei was not possible at day 6, because many of the cells had macroschizonts with a very large number of nuclei (300-500) closely packed together (see Fig. 2 B).

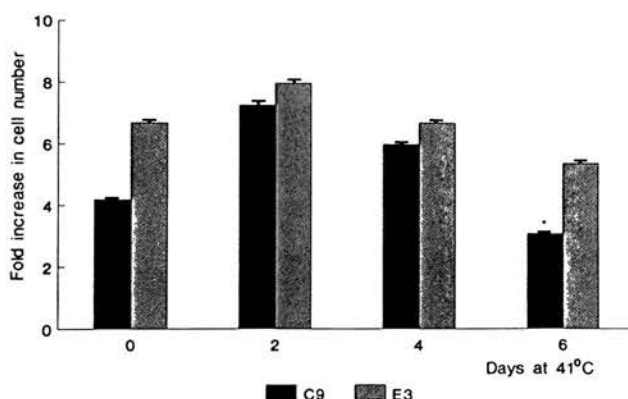
The increase in the mean number of macroschizont nuclei for the diminished cloned cell line E3 over the same period of time was found to be much smaller. Thus, from day 0 (37°C) to day 4 the mean number of nuclei increased by only 30%, from 13 to 17, and it took until day 12 for the mean number of nuclei to rise to a level of 32. Following prolonged (44 days) culture of these cells at 41°C, the mean number of nuclei fell to 11, a level not significantly different ( $P<0.01$ ) from the macroschizont-infected cells cultured at 37°C.

#### Comparison of infected cell growth at 41°C and 37°C

In order to analyse the growth rate of the cloned cell



**Fig. 5.** Mean number of nuclei per macroschizont after culture of cloned cell lines C9 and E3 at 37°C and at progressive 2-day time points after induction at 41°C. Filled bar, cloned cell line C9; hatched bar, cloned cell line E3. A dot above the column represents a significant difference ( $P<0.01$ ) between the means estimated at this and the preceding time point.



**Fig. 6.** Increase in cell number of cloned cell line C9 (filled bar) and cloned cell line E3 (hatched bar) over 48 h periods of culture. Days shown represent the value estimated at 37°C (day 0) and at 41°C (days 2-6). A dot above the column represents a significant difference ( $P<0.01$ ) between the means estimated at this and the preceding time point.

lines C9 and E3 during the first 6 days of differentiation at 41°C, cell counts were taken every 48 h. Fig. 6 shows that over the first 2 days of growth at increased temperature the amplification in cell number (7.3-fold) of the enhanced cloned cell line (C9) culture was greater than that estimated for the culture over 48 h at 37°C (4.2-fold). This level of growth at 41°C was not maintained, however, and as the time course progressed the increase in cell number of the culture steadily declined to 6-fold from day 2 to 4 and 3.1-fold from day 4 to 6. In general, the fluctuation in growth observed over the time course was similar for the culture of the diminished cloned cell line (E3). However, at each time point the estimated increase in cell number for the diminished cloned cell line was always greater than for the enhanced (see Fig. 6). For



example, after 48 h at 37°C the increase in cell number of the diminished cloned cell line (E3) was estimated at 6.7-fold, compared to 4.2-fold for the enhanced cloned cell line (C9).

## Discussion

We have isolated clones of *Theileria* macroschizont-infected cell lines that showed an enhanced or diminished ability to differentiate to the next stage in the parasite life cycle, the merozoite. Thus, we have established cell lines in which the majority of the parasites (>90%) differentiate over approximately the same time period. These cloned cell lines are a considerable improvement on cultures that have been used in previous studies of merozoite differentiation (Hulliger et al., 1966; Danskin and Wilde, 1976; Fritsch et al., 1988; Glascodine et al., 1990), and are essential for analysis of the molecular events that occur during merogony.

The percentage of cells of the enhanced cloned cell line (C9) that reacted with monoclonal 1D11 was found to be smaller than the number positive with monoclonal 5E1. In addition to this quantitative difference, monoclonal 5E1 produced a halo type pattern of immunofluorescence, whereas parasites that reacted with monoclonal 1D11 had a stippled pattern (see Fig. 3). Immunoelectron microscopic studies have shown that monoclonal antibody 5E1 binds to the surface of the differentiating macroschizont, whereas monoclonal 1D11 reacts with the rhoptry located within the differentiating parasite (L. Tetley and B. Shiels, unpublished data). We conclude that during the differentiation process the molecule detected by monoclonal 5E1 is expressed on the surface of the differentiating parasite before formation of the rhoptry is completed.

The reactivity of two of the monoclonal antibodies was found to be specific for either the enhanced (monoclonal 5E1) or the diminished (monoclonal 1C2) cloned cell line. Therefore, the epitopes recognised by these monoclonals are not conserved between the parasites of the different cloned cell lines, and the differentiating macroschizonts and merozoites generated from the two types of cloned cell line must be antigenically distinct. From these results it was predicted that the parasite genotypes represented by the different type of cloned cell line would be distinct, and this was confirmed by the result of the Southern analysis which showed a restriction fragment polymorphism between the enhanced and the diminished cloned cell lines (Fig. 4). Thus, like other stocks of *Theileria* (Allsop and Allsop, 1988; Conrad et al., 1989) our parental (TaA2) cell line contained at least two different parasite genotypes. These genotypes correlated with the enhanced or diminished differentiation phenotypes of the infected cell lines. This suggests that parasites of different genotype can have differing abilities to differentiate.

The results of the pulse experiment (see Table 3) showed that differentiation was not an immediate

response to the increased temperature, as the cells had to be in culture for more than 24 hours at the higher temperature before a proportion were induced to differentiate. Furthermore, it was evident that returning the cultures to 37°C reduced the level of differentiating cells from that obtained by continuing culture at 41°C for 7 days. From these results it appears that in order to differentiate the cells must go through a preliminary phase at 41°C, and that this early phase is reversible if the cells are returned to 37°C. However, returning the culture to the lower temperature after 4 days at 41°C did not result in a reduction in differentiation, when compared with the level observed immediately after 4 days of culture at 41°C (data not shown). Thus, we believe that, as in a number of other systems, including protozoan parasites (Watson et al., 1987; Bruce et al., 1990), the macroschizont once triggered becomes committed to an irreversible phase of differentiation that can proceed to completion at 37°C.

A characteristic that has been observed in the early stages of differentiation to the merozoite is an enlargement of the macroschizont, with a concomitant increase in its nuclear number (Hulliger et al., 1966; Glascodine et al., 1990). Our results show that the number of nuclei per macroschizont and the parasite size increased greatly during the first 6 days of culture of the enhanced cloned cell line C9 (see Fig. 5 and Fig. 2B), but with the diminished cloned cell line (E3) the increase was significantly smaller and took longer to occur. Furthermore, after 44 days of culture of the diminished line at 41°C the level of differentiation was very low (Table 2) and the mean number of nuclei per macroschizont was not significantly different from the number estimated at 37°C (Fig. 5). Thus, there is an association between the increase in the number of nuclei per macroschizont (and parasite size) and the ability of the cell lines to differentiate. We propose that this increase in parasite growth takes place during the initial reversible phase of the differentiation process until a condition is reached that triggers the second irreversible phase of differentiation to the merozoite.

In the growth analysis of the enhanced cell line (C9) we found, as postulated by Hulliger et al. (1966), that the growth of the macroschizont becomes asynchronous with the division of the host cell at 41°C. For the first 2 days the infected cell grew faster at the higher temperature (see Fig. 6). Therefore, in order to account for the increase in the number of parasite nuclei and size observed over this time (see Fig 5; cell line C9), the rate of parasite nuclear division must have been even greater than the increased host cell division. This increase in parasite nuclear division was then coupled with a decline in host cell division from two to six days, and it is likely that at a certain time point host cell division was inhibited completely. Owing to the association of the parasite with host cell spindle for separation of the macroschizont, as the rate of host cell division is reduced the division of parasites also becomes slower. Thus, an increase in parasite nuclear division/growth is accompanied by a decrease in



parasite cell division, and consequently the macroschizont is enlarged to the point where the host cell cytoplasm is completely filled (see Fig. 2B). The diminished cell line (E3) divided faster than the enhanced cell line at all time points tested, and the disruption of synchrony between parasite and host cell division was less at 41°C. This and the smaller size of the macroschizonts at 37°C probably caused the diminished phenotype of the E3 cell line, as it would take longer for the parasite to reach a predetermined size or condition which triggers differentiation.

Studies on the kinetics of replication of *T. parva* *in vivo* showed that an increase in the nuclear number of the macroschizont preceded the appearance of piroplasm infected erythrocytes. It was argued that macroschizont formation (i.e. differentiation to the merozoite) was time-dependent and was preceded by a fixed number of macroschizont-infected cell multiplications (Jarrett et al., 1969). The hypothesis of a mitotic clock regulating differentiation has been proposed for higher eukaryotic cells (Temple and Raff, 1986). Recent studies have demonstrated that the timing of such a clock is determined by control over proliferation, as in oligodendrocyte progenitor cells differentiation occurs prematurely when their proliferation potential is reduced by removal of growth factor (Raff et al., 1988). Moreover, differentiation can be inhibited by continued stimulation of the cells to proliferate by the addition of growth factors (Bogler et al., 1990). We believe the situation to be similar for differentiation to the merozoite, and that the timing of the initial reversible phase is regulated by the rate of increase in parasite size, with a concomitant decrease in the proliferation potential of both host cell and parasite.

In addition to studies carried out on higher eukaryotes, a correlation between a reduction in proliferation and differentiation has also been found for other protozoan parasites. For example, during its life cycle *Trypanosoma brucei* alternates between proliferative and non-proliferative phases (Vickerman, 1985), and repeated passage of the trypomastigote (bloodstream) stage can result in a loss of differentiation (Hajduk and Vickerman, 1981). In *Leishmania* differentiation from the insect promastigote stage to the mammalian infective stage (metacyclic) involves a change from logarithmic- to stationary-phase growth (Sacks and Perkins, 1984, 1985), and the inability of *Leishmania major* promastigotes to differentiate after transfer of an *in vitro* culture from 25°C to 35°C has been associated with proliferation at the higher temperature (Shapira et al., 1988). It is possible, therefore, that one of the parameters that leads to the triggering of stage differentiation in protozoan parasites is a reduction in the rate of parasite division, and that there are fundamental similarities between the mechanisms that regulate differentiation in parasites and higher eukaryotic cells.

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# Genomic and phenotypic diversity of Tunisian *Theileria annulata* isolates

L. BEN MILED<sup>1,2\*</sup>, K. DELLAGI<sup>1</sup>, G. BERNARDI<sup>1</sup>, T. R. MELROSE<sup>2</sup>,  
M. DARGHOUTH<sup>3</sup>, A. BOUATTOUT<sup>3</sup>, J. KINNAIRD<sup>4</sup>, B. SHIELS<sup>4</sup>, A. TAIT<sup>4</sup>  
and C. G. D. BROWN<sup>2</sup>

<sup>1</sup> Laboratoire d'Hématologie et d'Immunopathologie, Faculté de Médecine de Tunis, Tunisie

<sup>2</sup> Centre for Tropical Veterinary Medicine, Easter Bush, Roslin, Midlothian EH25 9RG, Scotland, U.K.

<sup>3</sup> École Nationale de Médecine Vétérinaire, 2020 Sidi Thabet, Tunisie

<sup>4</sup> Wellcome Unit for Molecular Parasitology, Faculty of Veterinary Medicine, Bearsden Road, Glasgow G61 1QY, Scotland, U.K.

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## SUMMARY

This study describes polymorphism in *Theileria annulata*, an intracellular protozoan parasite of bovine leucocytes and red blood cells. Fifty-three different stocks of *T. annulata*, isolated from 17 sites (districts) in Tunisia, have been characterized by anti-parasite monoclonal antibody (MAb) reactivity, glucose phosphate isomerase (GPI) isoenzyme electrophoresis, and Southern blotting with two genomic DNA probes. There appears to be considerable diversity amongst *T. annulata* stocks from Tunisia, no two isolates being identical, even those from animals on the same farm. Two distinct antigenic populations were detected by MAb 7E7. They were defined by negative and positive cells in the indirect fluorescent antibody test. The percentage of positive cells in different isolates ranged between 0 and 100%. The population variation seen by GPI analysis and DNA probes was greater; 7 different GPI phenotypes were identified amongst the stocks studied, while DNA probes *T. annulata* Tunis (*TaT*) 17 and 21 detected up to 5 different variants. The majority of isolates were shown to contain more than one parasite population, the number of variants per isolate ranging from 1 to 4. No correlation between particular parasite phenotypes or genotypes and their geographical site of isolation was observed. Selection of parasite populations *in vivo* and *in vitro* is also discussed.

Key words: *Theileria annulata*, polymorphism, DNA probes, isoenzymes, electrophoresis, monoclonal antibodies.

## INTRODUCTION

Tropical theileriosis caused *Theileria annulata* is widespread from Morocco, Spain and Portugal in the west, along the Mediterranean littoral through the Middle East and the Indian subcontinent to Central Asia (Purnell, 1978). In these countries tropical theileriosis is one of the most important threats to livestock production.

The parasite goes through three main stages: the invasive sporozoite transmitted by *Hyalomma* ticks; the intra-lymphocytic macroschizont and the intra-erythrocytic piroplasms in the bovine host. Macroschizont-infected cell lines have been used to attenuate the parasite by long-term *in vitro* culture and are currently used as the basis of vaccines (Pipano, 1977) that protect cattle in endemic areas. However, failures to protect fully have been attributed to the existence of variability in the parasite (Barnett, 1963; Pipano, 1979). Studies *in vivo* have shown that different field strains of *T. annulata* exhibit different levels of virulence in cattle (Pipano,

Weisman & Benado, 1974). A better understanding of virulence and protection might be provided by study of genotypically and phenotypically well-defined stocks which could also be used for cross-immunity trials and to provide potential candidates for vaccination.

At the present time it is not known which molecules are responsible for protection and therefore it is difficult to predict how cross-protective a given vaccine strain is likely to be, except by direct experimentation. Variation both within strains and between strains of *T. annulata* has been shown for a number of phenotypes and genotypes by studies on merozoite production *in vitro* (Shiels *et al.* 1992), monoclonal antibody profiles (Shiels *et al.* 1986), isoenzyme profiles (Melrose *et al.* 1983), a sporozoite surface antigen gene (Williamson *et al.* 1989) and merozoite/piroplasm antigens (Dickson & Shiels, 1993). It is clear from these studies that parasite variation is an important factor in the development of any subunit vaccine as well as in the production of vaccine strains for use in specific geographical regions.

To date most studies of variation in *T. annulata* have compared isolates from widely different geographical regions and little information is available

\* Reprint requests Dr L. Ben Miled, Centre for Tropical Veterinary Medicine, Easter Bush, Roslin, Midlothian EH25 9RG, Scotland, U.K.

### *B. Miled and others*

on the extent of parasite variation within a defined geographical region or whether particular variants are localized to specific areas within a region. This study is aimed at defining such levels of variation and therefore investigating, at a fundamental level, how important a factor parasite variation may be in the design of effective control measures. The diversity in *T. annulata* parasites is described using a combination of monoclonal antibody (MAb) reactivity, isoenzyme analysis and DNA probes. As a consequence, the study has extended the previous level of knowledge of diversity in *T. annulata*.

## MATERIALS AND METHODS

### *Parasite material*

**Derivation of stocks.** Fifty-three stocks, identified as *T. annulata* Tunisia (TaTu), were isolated from animals suffering from clinical theileriosis at 17 different sites (districts) in four bioclimatic zones of Tunisia (Fig. 1). They were numbered in chronological order of isolation, numbers being assigned to the farm from which they were isolated. Isolates from the same farm were given the same stock number followed by letters of the alphabet, each indicating a different isolate.

**Piroplasms.** Blood was collected from *T. annulata*-infected animals showing a piroplasm parasitaemia of between 5 and 7%. The infected erythrocytes were lysed using ammonium chloride solution (Martin, Finerty & Rosenthal, 1971) and the piroplasms were isolated as described by Conrad *et al.* (1987a).

***T. annulata*-infected cell lines.** Macroschizont-infected cell lines were established from peripheral blood mononuclear cells (PBM) isolated from cattle suffering from clinical tropical theileriosis and were maintained as described by Brown (1983). Cell clones were obtained by limiting dilution of macroschizont-infected cell lines. From these, five listed as clones 1, 2, 3, 4 and 5 are described in this study. Their respective parental cell lines were: TaTu 13 (clone 1), TaTu 9B (clones 2 and 3), TaTu 9A (clone 4) and TaTu 2 (clone 5).

**Experimental infection.** Four field isolates were passaged through animals by subcutaneous inoculation of  $5 \times 10^6$  macroschizont-infected cells from cell lines at low passage level ( $< 10$ ). These were TaTu 1 in calf 270, TaTu 2 in calves 29 and 164 and TaTu 9A in calf 170. Macroschizont-infected cell lines were established from these calves and piroplasms were isolated from calf 29. The animal number was used as a prefix to the stock number to indicate this; e.g. TaTu 1 in animal 270 is described as TaTu 270/1.

### *Monoclonal antibody reactivity*

MAB 7E7 used in this study was raised against macroschizont-infected cell line TaTu 270/1 using the method described by Pearson *et al.* (1980). It was used in the indirect fluorescent antibody test (IFAT) (Minami *et al.* 1983) to assess its reactivity with the macroschizont-infected cell lines described in this study.

### *Isoenzyme analysis*

Glucose phosphate isomerase (GPI) patterns were identified by thin-layer starch gel electrophoresis of lysates of piroplasms or macroschizont-infected cell lines as described by Melrose *et al.* (1984).

### *DNA preparation*

DNA was extracted from purified piroplasms, macroschizont-infected cell lines and uninfected bovine PBM using standard methods (Maniatis, Fritsch & Sambrook, 1982; Conrad *et al.* 1987a).

### *Construction and screening of the genomic library*

DNA extracted from purified piroplasms of TaTu 7 was digested with restriction endonuclease *Eco*R1 according to the manufacturers' specifications (Boehringer, Mannheim) and cloned into the *Eco*R1 sites of pUC18 as described by Maniatis *et al.* (1982). Twenty-one resulting recombinants, which did not hybridize with bovine DNA, were studied further. Insert lengths ranged from approximately 0.92 to 16 kilobases (kb). Three of the recombinants contained two *Eco*R1 fragments, one contained three and all other recombinants showed a single fragment as defined by *Eco*R1 digestion. Two recombinants designated *T. annulata* Tunis (TaT) 17 and 21 with inserts of 7 and 16 kb, respectively, were selected for use as probes in hybridization analysis. The TaT 21 insert contained two *Eco*R1 fragments of 7.9 and 8.1 kb, and TaT 17 a single fragment.

### *Southern blotting*

Samples of 2–4 µg of purified piroplasm DNA and 10–20 µg of macroschizont-infected cell line DNA or uninfected bovine PBM DNA were digested with restriction endonuclease *Eco*R1 as specified by the manufacturer (Boehringer, Mannheim) and were then subjected to electrophoresis through 0.7% agarose gels (Maniatis *et al.* 1982). The gels were blotted (Southern, 1975) onto nylon membranes (Hybond N, Amersham) and the DNA fixed by exposure to ultraviolet light for 5 min and kept at  $-20^\circ\text{C}$  if not used immediately. The filters were



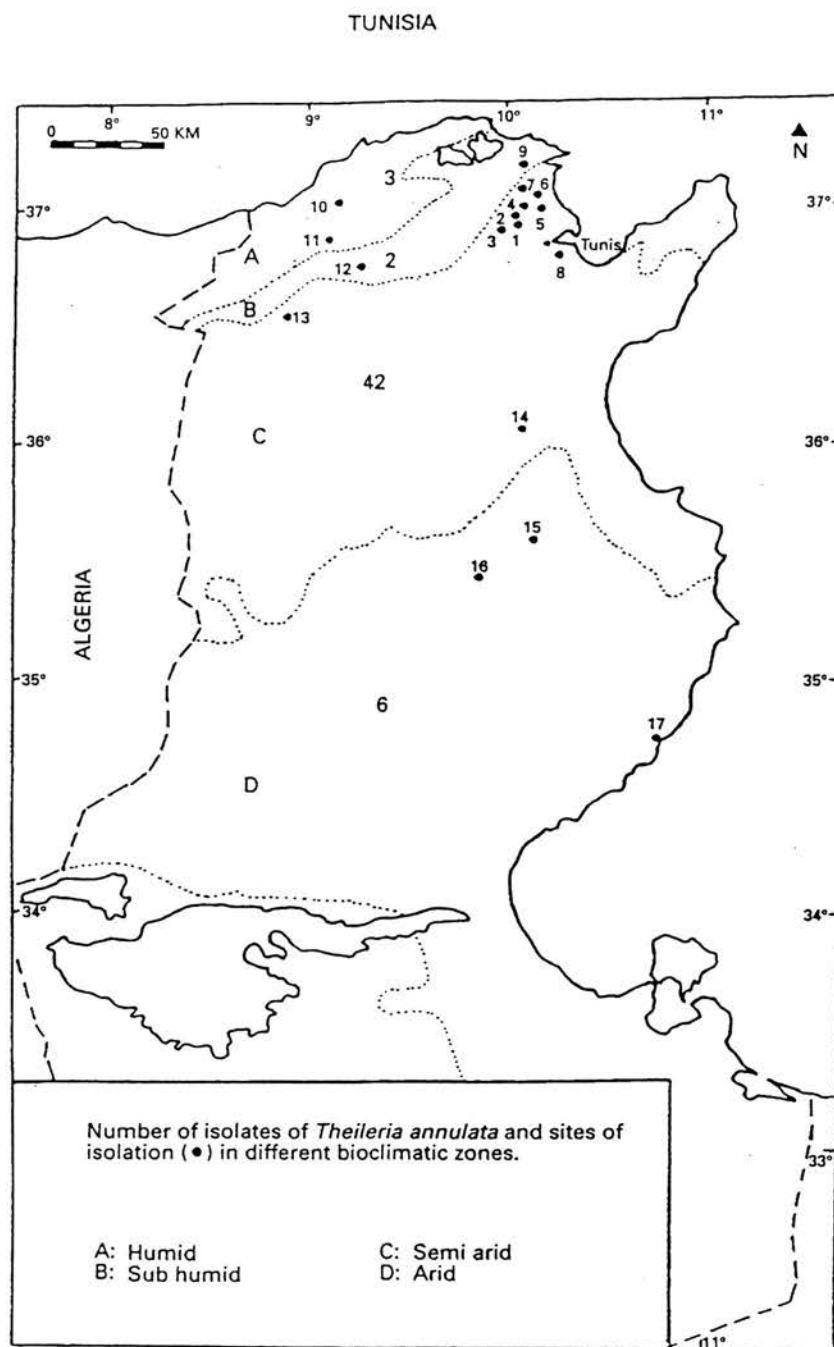


Fig. 1. Map of Tunisia showing number of isolates of *Theileria annulata* (large numbers) and sites of isolation (small numbers) in different bioclimatic zones.

hybridized using the selected probes and washed at high stringency (40 mM  $\text{Na}_2\text{HPO}_4$ , 1% SDS) at 65 °C, by the method of Church & Gilbert (1984). The probes were labelled with  $(^{32}\text{P})\text{dCTP}$  by random priming using a commercial kit (Boehringer, Mannheim). Filters were autoradiographed at -70 °C for between 2 h and 5 days.

## RESULTS

This is the first report, using three different methods of analysis, describing polymorphism in *T. annulata* stocks from one geographical region. Samples were

collected from 17 different sites in 4 bioclimatic zones (Fig. 1) within Tunisia and a total of 53 *T. annulata* stocks were isolated.

In order to examine and characterize the phenotypes and genotypes of these stocks, fixed slide preparations of the macroschizont-infected cell lines were made for IFAT using MAb 7E7, protein extracts were prepared for starch gel electrophoresis and the detection of GPI and DNA was extracted from each of the stocks for Southern blotting using the probes *TaT17* and *TaT21*. Thus each stock was characterized in terms of the level of variation with respect to each of the markers described above. The

Table 1. Comparison of *Theileria annulata*-infected cell lines isolated from different bioclimatic zones (A-D)

TaTu stocks	Site of isolation	MAb 7E7 reactivity (%)	GPI types	TaT 17 profiles	TaT 21 profiles
Zone A					
37	10	100	1+4	1+2+4	1+2
38	11	100	4+6	3+4+5	1+3
39	11	100	2+4	5	1
Zone B					
35	9	0	6	3	1
36	12	15	2+4+7	3+7	1
Zone C					
19	4	100	4	3	3
20		100	2+4+6	3	1
21		100	2+4+6	3+5	1
22		100	4	5	1
23		100	2+4	2	1+3
25	6	100	4	2	1+3
27		100	2+6	2+3	2
28	6	50	2+4	3	1
29		100	6	2+4	1+3
30		100	2+4	3+5	1+2+3
31		15	4+6	3+4	1
32		40	2+4	3+5	1+2
40	13	20	1+4+7	3+5	1+2
41		20	2+4	3+5	1
42		0	2+5	2+3	1
43		10	2+5	2-3	1
44		100	1+4	2	1
45		50	2+4	2+3+5	1
Zone D					
49	15	40	4	2+3+4+5	1+2+3
46	16	100	4+6	4	1
47	16	30	4	2+3	1
50	16	50	2+4	3+5	1+2
51	17	100	2+4+6	2+3	1+(2)+(3)*

\* ( ) Faint bands.

results are summarized in Tables 1-4, although not all stocks collected from Zone C are presented due to their large number.

MAb 7E7 was selected due to its pattern of IFAT reactivity with macroschizonts in cell line TaTu 270/1 and its lack of reactivity with uninfected bovine PBM. This MAb was then tested on macroschizont-infected cell lines and recognized only 1% of schizonts in infected cells in stock TaTu 2 but reacted with 100% of schizonts in stock TaTu 9A. This initial investigation suggested that MAb 7E7 detected antigenic diversity in *T. annulata* stocks and was therefore chosen to screen further macroschizont-infected cell lines by IFAT. Variation in the percentage of macroschizonts recognized by MAb 7E7 in the infected cell lines, was observed with the proportion of positive cells ranging from 0 to 100%. When cell lines showing intermediate percentages of staining were cloned by limiting dilution, the cloned cell lines segregated into either totally negative cell lines (0% positive cells: 7E7-) or

totally positive cell lines (100% positive cells: 7E7+). The cell lines in which only a proportion of the macroschizonts were positive can be interpreted as consisting of a mixture of the two phenotypes 7E7+ and 7E7-.

The stage specificity of the expression of the antigen recognized by this monoclonal was determined by demonstrating that the MAb reactivity with intra-erythrocytic piroplasms was always negative even when the cell line from the same isolate was 100% positive. This suggests that the antigen is stage-specifically expressed in the macroschizont.

The results of analysis of isolates from different regions are shown in Table 1. Zone A is homogenous for the 7E7+ phenotype which is relatively rare in Zone B but occurs in Zone D at a high frequency. Analysis of different isolates from the same sites in Zone C showed a similar level of 7E7 polymorphism as that found between other zones (Table 1). Site 4 appeared homogeneous for the 7E7+ type whereas this phenotype occurred at variable frequencies in

Table 2. Comparison of *Theileria annulata*-infected cell lines isolated from animals on the same farm in zone C

TaTu stocks	Site of isolation	MAb 7E7 reactivity (%)	GPI types	TaT 17 profiles*	TaT 21 profiles
9	A 3	100	2+4	(2)+3+4+5	1+2
	B	100	2+4	2+3+4+5	1+3
14	A 1	0	4+6	3+(4)	2
	B	0	4	5	1
	C	15	4+6	4+5	1
18	A 4	100	1+5	3+4	3
	B	100	4	3+5	2
	C	100	2+4+6	3	1
24	A 3	1	6	3	2
	B 3	25	2+4+6	(2)+3+5	1+2

\* ( ) Faint bands.

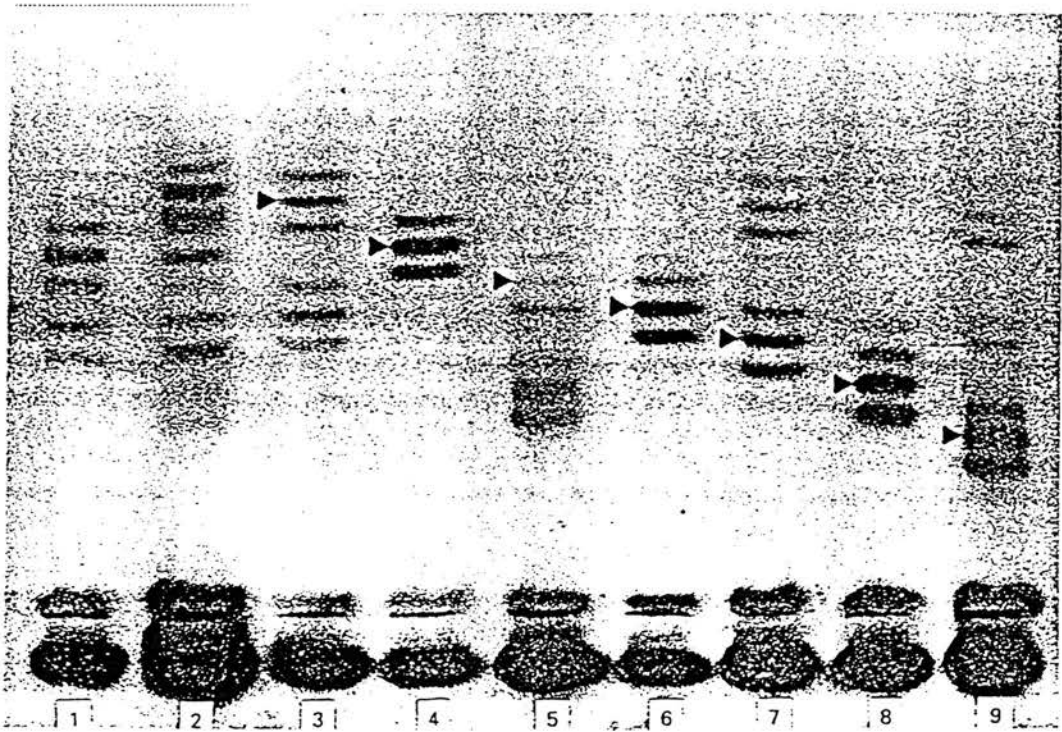


Fig. 2. GPI isoenzyme patterns of *Theileria annulata*-infected cell lines. Lanes correspond to stocks: Lane 1, TaTu 50 (2+4); lane 2, TaTu 11 (1+2+4+6); lane 3, TaTu 37 (1+4); lane 4, TaTu 27 (2); lane 5, TaTu 16 (3+6); lane 6, TaTu 22 (4); lane 7, TaTu 18A (1+5); lane 8, TaTu 29 (6); lane 9, TaTu 40 (1+4+7). (Arrows show each of the GPI types.)

different isolates in sites 6 and 13. When multiple isolates were taken from single farms (Table 2) in Zone C, the same variation was seen within a farm. Some farms were homogeneous while others showed a range of proportions of the two variants.

Polymorphism in the isoenzyme pattern for glucose phosphate isomerase (GPI) has been shown to occur between isolates from different countries (Melrose, 1983; Melrose *et al.* 1984) and it has been shown that a cloned isolate possesses a three-banded electrophoretic pattern (Wilkie *et al.* 1984). When the 53 isolates were examined for their GPI phenotype the majority showed a multiple triplet

band pattern. Seven variant forms of GPI triplets were identified (Fig. 2). They were classified according to the extent of their migration from the most anodal (1) to the most cathodal (7). On the basis of the three-banded patterns given by individual clones, each multiple banded type has been scored accordingly to its composite GPI pattern. Hence TaTu 11 with four triplet types was scored as 1+2+4+6.

When zones A, B, C and D (Table 1) were examined it could be seen that the majority of isolates were mixtures. The complete range of 7GPI variants was only found in zone C, and included

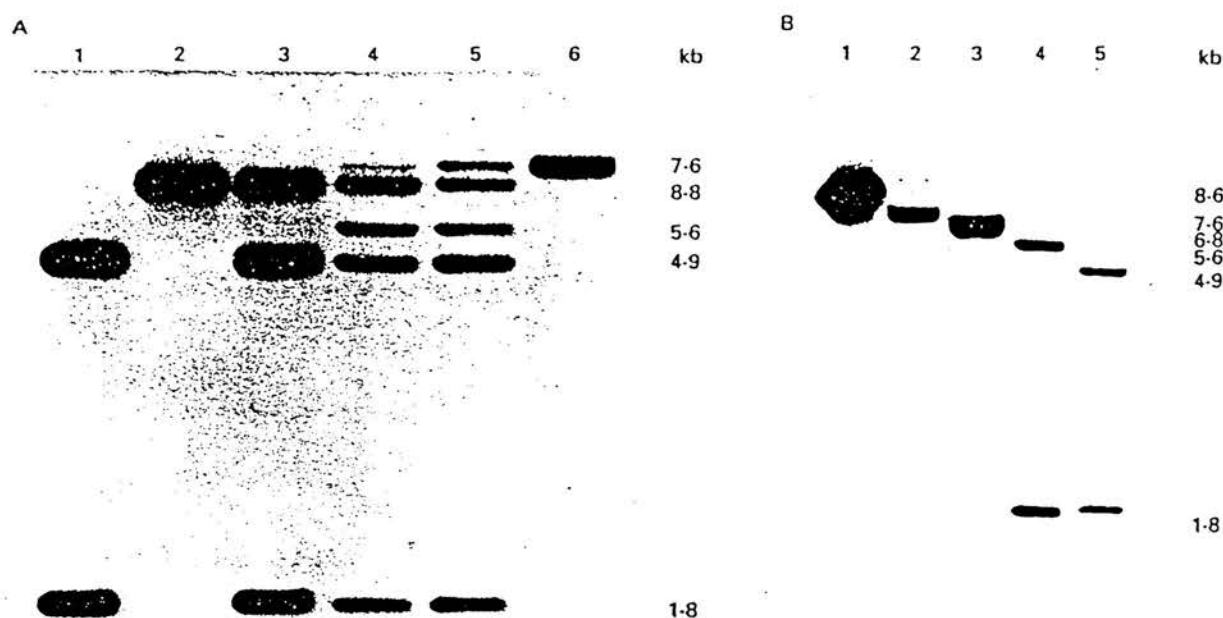


Fig. 3. Southern blots of *Theileria annulata* DNA digested with *Eco*RI and hybridized with probe *Ta*T 17. Lanes correspond to stocks. (A) *T. annulata* piroplasms: Lane 1, *Ta*Tu 270/1; lane 2, *Ta*Tu 7; lane 3, *Ta*Tu 29/2; lane 4, *Ta*Tu 9A; lane 5, *Ta*Tu 9B, lane 6, *Ta*Tu 10. (B) Clones of *T. annulata*-infected cell lines: Lane 1, clone 1; lane 2, clone 2; lane 3, clone 3; lane 4, clone 4; lane 5, clone 5.

variant 3 which was detected once in cell line *Ta*Tu 16 (Fig. 2, Lane 5), isolated from site 5. Isolates which were homogeneous on the basis of the 7E7 epitope could have composite GPI patterns. A similar pattern of variation was found when isolates from the same site were compared (Table 1) and also when several isolates from the same farm were compared (Table 2).

Polymorphism at the level of the genome within and between isolates was examined using two polymorphic gene probes (*Ta*T 17 and *Ta*T 21). Probe *Ta*T 17 identified five different genotypes present either as a single profile or as a mixture of profiles of different variants (Fig. 3A). The five profiles obtained using DNA from cloned macro-schizont-infected cell lines, respectively clones 1–5, probed with *Ta*T 17 (Fig. 3B), suggest that the gene fragment is single copy in the genome and varies in both size and the number of *Eco*RI sites it contains. No internal *Eco*RI site was detected in probe *Ta*T 17 which derived from the *T. annulata* stock *Ta*Tu 7 used to generate the genomic library. DNA from this stock showed a single *Eco*RI fragment (7 kb), a result consistent with the lack of an internal *Eco*RI site (Fig. 3A, Lane 2). When DNA from clones 1, 2 and 3 was probed with *Ta*T 17, each showed a unique fragment of respectively 8.6, 7.6 and 6.8 kb (Fig. 3B). DNA from clones 4 and 5 showed bands of 5.6 and 4.9 kb respectively, and an additional 1.8 kb fragment in both, which presumably indicates that the locus detected by this probe contains an additional *Eco*RI site spanned by probe *Ta* 17.

Probe *Ta*T 21 contains an internal *Eco*RI site and the results were therefore less straightforward to analyse than for probe *Ta*T 17. Profiles, composed of three basic patterns of a 7.8 kb fragment (profile 1), 4.3 and 3.9 kb fragments (profile 2) and 4.5 and 3.7 kb fragments (profile 3) were detected by the *Ta*T 21 as illustrated by Fig. 4. DNA from clone 5 (as defined by probe *Ta*T 17) probed with *Ta*T 21, showed both a 7.8 kb fragment and the 4.3, 3.9 kb doublet (Fig. 4B). This might indicate that clone 5 was not a clone.

Based on the analysis of the isolates from different zones (Table 1), it can again be seen that the majority of the isolates are mixtures of different genotypes and that the same variants were found in different zones; variants 1, 2 and 4 (*Ta*T 17) were not found in zone B. However, only two isolates were examined from this zone. Similarly extensive polymorphism was found between isolates from within a site and within a single farm (Tables 1 and 2).

In some cases more than one marker was required to detect the full level of diversity within a stock. For example, stock *Ta*Tu 49 with a single GPI profile, clearly consisted of several populations when DNA probes were used (Table 1). Likewise when DNA from stock *Ta*Tu 13, was probed with either *Ta*T 17 or 21, the piroplasms and macro-schizont-infected cell line showed identical profiles, but GPI analysis of this stock demonstrated differences between them in that the piroplasm population was of single profile type 4 while the macro-schizont-infected cell line was of single profile type 6 (Table 3).



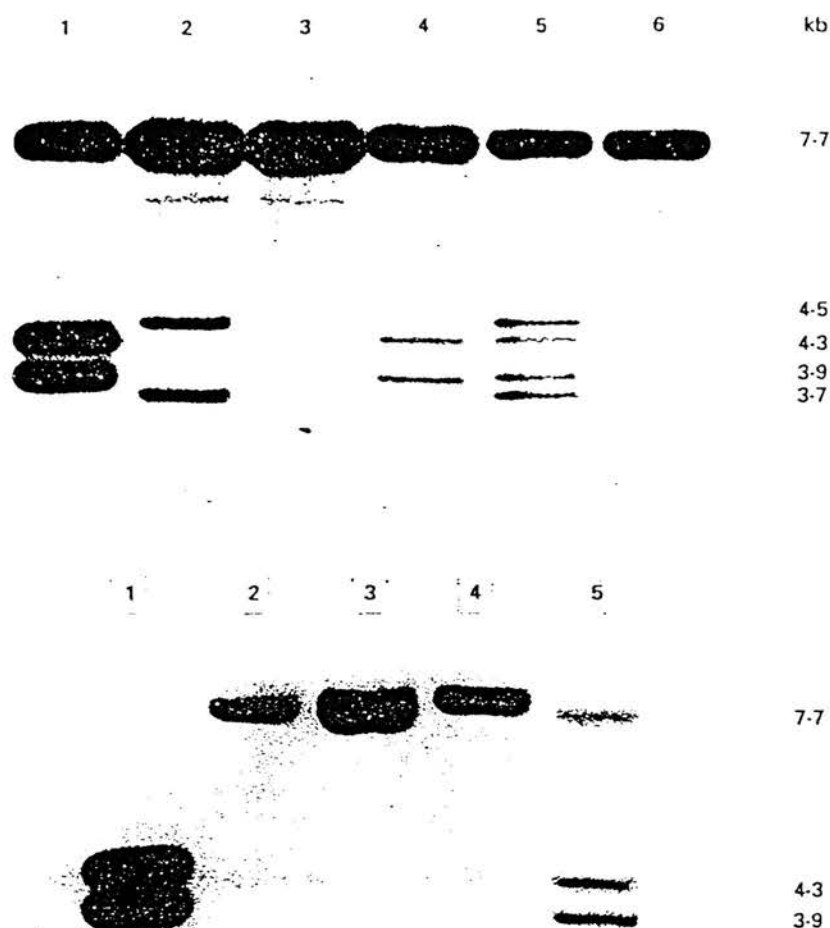
Polymorphism in *Theileria annulata*

Fig. 4. Southern blots of *Theileria annulata* DNA digested with restriction enzyme *Eco*RI and hybridized with probe TaT 21. (A) *T. annulata* piroplasms. Lanes correspond to stocks listed in Fig. 3A. (B) Clones of *T. annulata*-infected cell lines. Lanes correspond to clones listed in Fig. 3B.

Although, the majority of isolates were shown to contain a mixture of genotypes or phenotypes, a few isolates appeared to consist of a single population, e.g. TaTu 35, TaTu 42 (Table 1), based on analysis by all three types of marker.

The material used for this study was mainly derived from macroschizont-infected cell lines. This raises the question as to whether such a system provides an accurate representation of the parasite variants within the individual animals sampled. Two approaches were undertaken to examine this.

First, a comparison was made between the GPI and DNA probe profiles of piroplasms and macroschizont-infected cell lines, isolated from the same animal at the same time. The results of this analysis are presented in Table 3 for isolates from zone C. It is clear that the variants defined could differ depending on whether macroschizonts or piroplasms were examined. In three of the samples (14C, 18A

and 18C) a larger number of variants were detected in the piroplasms than in the macroschizont-infected cell line derived from the same animal, suggesting that some type of selection reducing diversity may be occurring in culture. However, the data from isolate TaTu 11 do not support this, as the cell line showed greater heterogeneity than the piroplasm preparation. Clearly, the method of isolating the parasite is important and requires further investigation, although if the selection observed was random with respect to the different variants it should not affect the analysis.

Secondly, and to examine this question further, three calves were infected experimentally, each with a cell line containing a mixed parasite population, piroplasms and cell lines were subsequently obtained from them and typed as described previously. The results showed that overall the number and identity of variants recovered in piroplasms differed to those

Table 3. Comparison of *Theileria annulata* macroschizont-infected cell lines (CL) with their homologous piroplasms (P)

<i>T. annulata</i> stocks	Parasite material	GPI types*	TaT17 profiles*	TaT21 profiles
11	CL	1+2+4+6	3	1
	P	2	3+(4)	1
13	CL	6	1	2
	P	4	1	2
14C	CL	4+6	4+5	1
	P	2+4	3+5	1+2
18A	CL	1+5	3+4	3
	P	1+2+4	3+4+5	1+2+3
18C	CL	2+4+6	3	1
	P	(1)+2+4+6	3+(5)	1+2+3

\* ( ) Faint bands.

Table 4. Comparison of *Theileria annulata* stocks isolated from experimentally infected calves (\*) with their parental cell lines (\*)

TaTu stocks	Parasite material†	MAB 7E7 reactivity (%)	GPI types	TaT17 profiles‡	TaT21 profiles‡
•2	CL	1	1+4	3+5	1+(2)
•29/2	CL	0	4	5	1
	P	—	4	3+5	1
•164/2	CL	0	4	5	1
•9A	CL	100	2+4	(2)+3+4+5	1+2
•170/9A	CL	100	4	(3)+5	1
	P	—	4	3+5	1

† CL, Cell line; P, piroplasms.

‡ ( ) Faint bands.

found in the cultured cell lines (compare CL with P profiles, Table 4). The differences between the profiles of the cell lines and the profiles of the piroplasms (derived from these calves) were similar to those found when the same comparison was made using naturally infected animals (Table 3) as described above. In addition, not all the variants present in the cell line used for infection were recovered from the infected calf in either the piroplasms or the cell lines.

#### DISCUSSION

The aim of this study was to monitor the extent of heterogeneity in *T. annulata* in Tunisia. The data presented shows that considerable polymorphism exists amongst the parasite population within this single country. As the genome of *T. parva* is known to be haploid (Morzaria *et al.* 1992), it is likely that the situation would be the same for *T. annulata*. Therefore the variation seen in these stocks is almost certainly due to the presence of mixed parasite populations which could be separated by limiting dilution cloning. Up to four variants within one parasite stock were detected by either by GPI

electrophoresis, e.g. TaTu 11 (Table 3) or genomic analysis e.g. TaTu 9A (Table 2). Using all four markers no two isolates had the same profile, even when isolates from animals on the same farm were examined (Table 2), nor was there any correlation between the marker profiles of isolates from the same site (Table 1). Previous studies have shown variation between *T. annulata* populations from different countries using MABs (Shiels *et al.* 1986). The presence of mixed parasite populations have already been reported for *T. annulata* (Shiels *et al.* 1986; Williamson *et al.* 1989) and for *T. parva* (Toye *et al.* 1991). Polymorphism in *T. parva* stocks was detected using phenotypic (Pinder & Hewett, 1980; Minami *et al.* 1983; Conrad *et al.* 1987b) and genotypic analyses (Conrad *et al.* 1987a, 1989; Allsopp & Allsopp, 1988). The studies on *T. annulata* only analysed a limited number of isolates (3–12); however, the present work has examined *T. annulata* parasite populations using a large number of isolates (53) from a single geographical region and the analysis was carried out by both phenotypic and genotypic markers.

Diversity was detected between the isolates and between different parasite stage samples from the

same animal; for example, between macroschizont-infected cell lines and their derived piroplasms. In general, more variants were detected in piroplasms than in macroschizont-infected cell lines using genomic probes. This could be explained by the selection of parasite populations during the establishment of the cell lines *in vitro*. However, with some isolates the opposite was clear from GPI analyses. This was well illustrated by the isolate TaTu 11 where four GPI types were detected in the macroschizont-infected cell line but only one in the piroplasms. It has been found that cloned macroschizont-infected cell lines have differing ability to differentiate to the merozoite stage *in vitro* (Shiels *et al.* 1992). The subpopulations which are not present in the piroplasm preparation might be those which have a diminished ability to differentiate and infect erythrocytes, producing a low level of piroplasms of this type. Alternatively, selection for minor populations which are not observed in the piroplasm sample could be occurring in the generation of the cell line. The origin of this variation in marker profiles needs to be examined in the future and the question of whether the divergence of marker profiles between different stages of the parasite is due to selection within the host or in culture could be examined using PCR and oligonucleotide primers specific for each allele.

From experimental infection *in vivo* with macroschizont-infected cell lines, it was noticed that changes occurred in both the relative proportions and the type of variants recovered from the infected animals. One identical variant was present in cell lines isolated from animals 29 and 164 as assessed by probe TaT 17 and GPI analysis, whilst in the parental cell line (used for infection) two variants were detected by each marker. However, when piroplasm DNA from TaTu 29/2 was probed with TaT 17 both genotypes were present. This could suggest either that the establishment of particular variants is easier *in vivo*, or that during *in vitro* culture this type grows more successfully. Both hypotheses could be correct since in animal 29 the two variants present in the parental cell line successfully established *in vivo* as demonstrated by piroplasm analysis, but only one was detected in the *in vitro* established cell line. On the other hand, from animal 170, infected with the TaTu 9A cell line, only two out of four variants originally present in the parental stock, as assessed by probe TaT 17, were detected in both the piroplasms and the macroschizont-infected cell line. Therefore the weakness of this study is the difficulty in obtaining all the populations originally present in one isolate after *in vitro* culture and also the limited amount of homologous piroplasms which can be obtained from field isolates.

The polymorphism between and within *T. annulata* stocks showed no correlation with the geo-

graphic location of the isolates, even those made from animals on the same farm were found to differ. Similar results have been reported for *P. falciparum* isolates from patients in one village where even isolates from the same family were of different genotypes (Babiker *et al.* 1992). The results also raise a concern for future studies in that the possibility of selection occurring through manipulation *in vitro* or *in vivo* should always be recognized when interpreting data to characterize *T. annulata* parasites. As large numbers of clones have not been established from each isolate it was not possible to determine the numbers of distinct genotypes within the population but, if the four markers used are unlinked, there may be a very large number within an individual animal. These results are in contrast to the findings of Matsuba *et al.* (1992), on *T. sergenti*, who reported identical genomic patterns using two DNA probes for piroplasms DNA preparations from animals grazing in the same area, although polymorphism was observed between stocks isolated from different areas. The mechanism of selection described here could also account for the disappearance of a genomic fragment in Southern blots, when stocks are passaged *in vitro* in the study of Matsuba *et al.* (1992).

The next step in our investigation will be to provide an explanation for such diversity. Since genetic recombination occurs in *Plasmodium falciparum* (Ranford-Cartwright *et al.* 1991) and has now been reported for *T. parva* (Morzaria *et al.* 1992) it is likely that it might generate the tremendous polymorphism seen in *T. annulata*.

Diversity within individual parasite populations would seem to be an inherent facet of infection by *T. annulata* and has important implications for a successful vaccination programme using either a live attenuated or recombinant subunit vaccine, if the antigen generating immunity showed a similar level of diversity.

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